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Demands of a Theory for an Aging Mechanism involving a Soft and Hard Electrophile

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Abstract

Organisms from cyanobacteria to humans, are equipped with oxidoreductases, conserved from cyanobacteria to humans, that serve as light receptors and play roles in electron transfer in detoxification, respiration, metabolism and biosynthetic processes. These oxidoreductases include enzymes that contain flavins, pterins and cytochromes, which participate in blue light input pathways and subject to circadian clock control, and contain binding sites for $NAD(P)^+$, an electron mediator. The endogenous quinonoid indole-2,3-dione, which exhibits a bright orange colour due to blue light absorption and undergoes circadian control, has long been identified among plants and bacteria as a participant in metabolic pathways catalyzed by enzymes, that are not present in animals. Indole-2,3-dione interferes with the binding of ATP or NAD(P)H to atrial natriuretic peptide receptors, peripheral benzodiazepine receptors and blue light receptor flavoenzymes and demonstrates dual reactivity toward glutathione (GSH)/proteins and DNA/RNA as both a hard and soft electrophile. The formation rate of indole-2,3-dione is dependent on the activity of tyrosine hydroxylase and may affect cell fate, GSH status, $NAD(P)^+$-linked oxidation, cytochrome functions and ADP/ATP-linked energy conservation through caspase 3 inhibition or carbon monoxide liberation. This quinonoid, which is a plant growth hormone and is capable of single-electron transfer, may play a central role in the aging mechanisms of animals as well as in age-associated disease states.

Keywords: Detoxification; Oxidoreductase; Bacteria; Oxygen; Lucigenin; Aging

Introduction

Organisms are continuously challenged by numerous exogenous and endogenous stressors from embryogenesis through death. A series of stress responses followed by recovery to equilibrium occurs throughout life. Overall, regulatory mechanisms governing the stress response in animals, involving the integration of the neuroendocrine and immune systems, have been designated the sympathoadrenomedullary system and the hypothalamohypophysial system. Stress responses begin with a metabolic burst in the nervous system that is energetically driven by robust oxygen consumption in the mitochondria, permitting the generation of electrophiles and free radicals that mediate electron-borne information or otherwise must be detoxified. Enzymatic detoxification of xenobiotics and nutritionally useless electrophiles is thought to constitute a primordial stress response, descending from unicellular organisms that were capable of an enormous range of catabolic activity. However, animals have evolved detoxifying enzymes that, while adapted for elimination, possess much less specificity for various compounds, rather than the high specificity observed in bacteria [1]. Organisms are equipped with a number of oxidoreductases, which are highly conserved from phototrophs to humans, that are utilized throughout detoxification, metabolism, biosynthesis and respiration. Oxidoreductases are enzymes containing flavins, pterins or cytochromes and in many cases utilize free $NAD(P)^+$ in addition to protein-bound FAD to mediate electron-borne information. These enzymes, which are capable of absorbing blue light, are components of circadian light input pathways and are themselves under clock control, suggesting they constitute a primordial stress response to light. The activities of blue light receptor enzymes are indispensable for synchronization with the environment and circadian clocks, comprising a rhythm generator that responds to specific signals (*zeitgeber*). NAD(P)H, the ubiquitously occurring electron donor that is conserved in both anaerobic to aerobic cells, has been implicated as a mediator of electron transfer in both short-period and circadian clocks particularly in light input pathways.

In animals, incomplete elimination of electrophiles that interfere with blue light reception as well as attack nucleophilic macromolecules can result in the disruption of electron-borne information associated with alterations to redox states, intracellular pH and the metabolism of nitrogen and sulfur, leading to subtle changes in the outcome of the stress response, which would otherwise terminate at original equilibrium. This sequence of events may be accompanied by a decline in respiratory function in the mitochondria. Mechanisms of aging may involve the deteriorating maintenance of the equilibrium between the stress response and detoxification.

It is conceivable that sulfur-containing tripeptide glutathione(GSH) is a strong candidate as a link between the stress response, detoxification and aging because it confers increased resistance to stress in early embryos as early as the 2-cell stage, when increased production of the heat shock protein (HSP)70 family also manifests [2], and detoxifies redox-cycling compounds and electrophiles that cause DNA damage and protein modifications throughout life additionally, GSH metabolizes hydrogen peroxide, which constitutes the only available defense against this molecule, particularly in the mitochondria. Diurnal variations in GSH have been observed in the nervous system and liver and are closely related to the oxidative stress cycle [3-6], strongly suggesting a reciprocal relationship between the stress responses in the liver, a major reservoir of the systemic GSH pool, and the nervous system, an organ with the highest rate of oxygen uptake and lipidperoxidation. Stress-

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induced increase in oxygen consumption in the central and peripheral nervous system modulates GSH status via electrophile-mediated mechanism(s), which in turn, partly via altered redox states, affect gene expression, cell fate and cycle and membrane transport, with the most profound effects being observed for the nervous system due to the high rate of electrophile production.

Circadian clocks, found in all phyla from cyanobacteria through humans comprise a rhythm generator as well as inputs and outputs. Light is a strong signal from the environment for all circadian systems. Blue-light receptor enzymes may also be components of a primordial form of stress response. Diurnal variations in animals have also clearly been observed in $NAD(P)^+$-utilizing oxidoreductases, such as NO-Synthase (NOS), Monoamine oxidase (MAO) and Xanthine oxidase (XO), as well as in adenosine, suggesting that NAD(P)H liberates electrons towards these enzymes during the application of light. Disrupted inputs should accompany altered outputs, including intracellular redox states, pH, and nitrogen and sulphur metabolism. GSH status may reflect diurnal variations in electron-borne information underlying detoxification, biosynthesis and respiration.

For a proper theoretical model of aging mechanism to exist, the demands of such a theory should be as follows:

(1) Aging should be accounted for in a fundamental cycle that occurs in succession from embryogenesis through death because aging originates in embryogenesis and is merely an aspect of development (Figure 1).

(2) A fundamental cycle should be based on the concept of stress response and involve inflammatory processes because age-correlated disease states are also stress-related and involve inflammation (Figure 1).

(3) The mechanism underlying the increasing incidence of age-correlated disease states must align with a single mechanism of aging. Any manifestation at the initiation of age-related disease states should align with a single mechanism of aging (Figure 2).

(4) Quantitative changes to one or more constituents of a fundamental cycle must directly or indirectly cause qualitative changes leading to any manifestation of age-correlated disease states. In other words, a distinct quantitative threshold should exist for a component of a fundamental cycle to form a biologically active substance that causes a qualitatively distinguishable state associated with age-correlated diseases (Figure 3).

According to the mitochondrial theory of aging [7-9], the accumulation of mutations in mitochondrial DNA (mtDNA) in somatic cells due to continuous attack by toxic oxygen species is a key factor determining the selective impairment of acceptor substrate binding to subunits encoded by mtDNA, and enzyme inhibition of complex I leads to the decline in cell energetics that characterizes senescence.

Important problems left unsolved may be as follows:

(1) Why is overall NAD+-linked oxidation more severely compromised in aging than complex I activity?

(2)In aged mitochondria, from what is the distinct variability in the presence and extent of functional deterioration of respiratory enzyme activities derived?

(3)Why does decreased rotenone sensitivity for complex I, which is indicative of the specific deterioration of physiological quinone (substrate) binding with age, precede the disruption of electron transfer?

(4) How can the association between complex I disruption throughout the body in cells of any developmental origin and the selective vulnerability of catecholaminergic terminal regions both in the periphery and in the brain, including the hippocampus, striatum and salivary glands, in aged animals be rationalized?

(5) How does the mitochondrial theory align with a mechanism explain the age-correlated increase in susceptibility to diseases when the presence and the extent of selective catecholaminergic cell

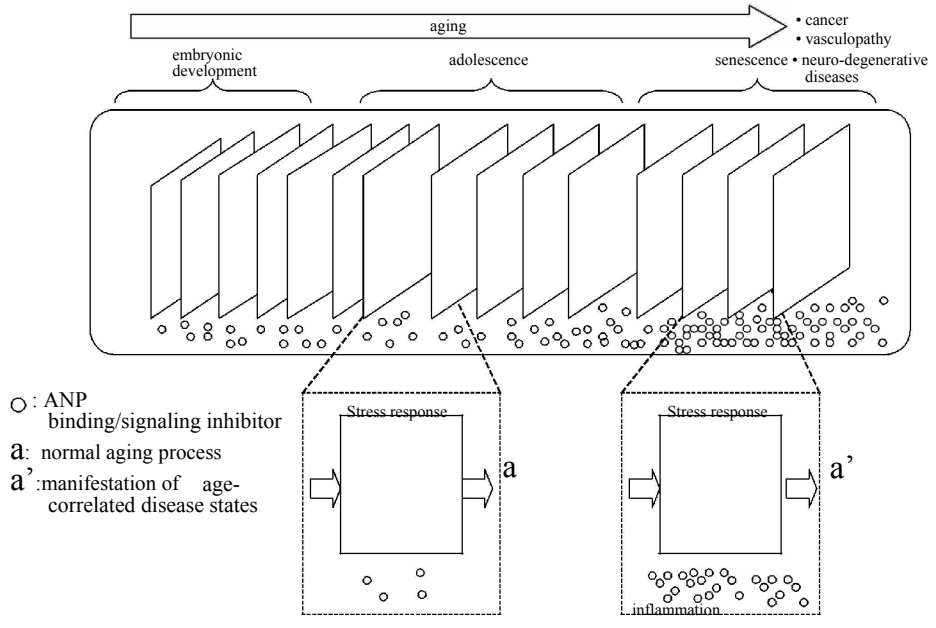

Figure 1: A theoretical model of aging mechanism: a fundamental cycle in succession constitutes whole life. A fundamental cycle consisting of NO synthase and TH activities represents stress response and constitutes whole life. Increase in ANP binding/signaling inhibitor, indole-2,3-dione, is associated with inflammation.

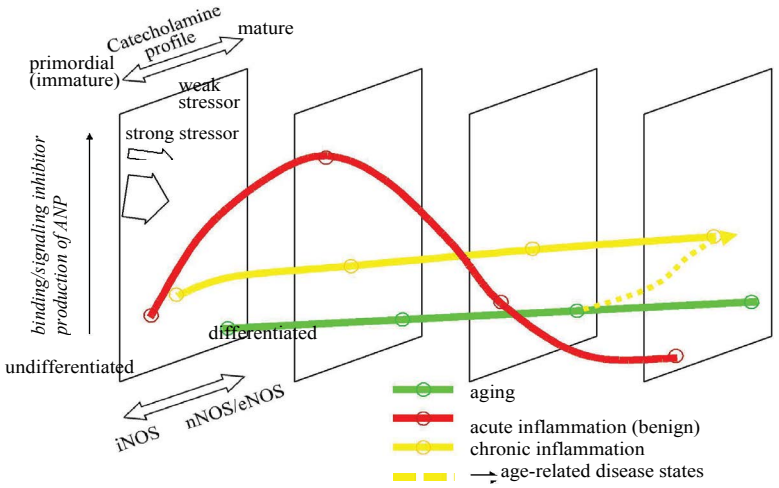

Figure 2: Schematic drawing of patterns of formation for ANP binding/signaling inhibitor. The dotted line indicates the way how age-correlated diseases is increasing in incidence with age.

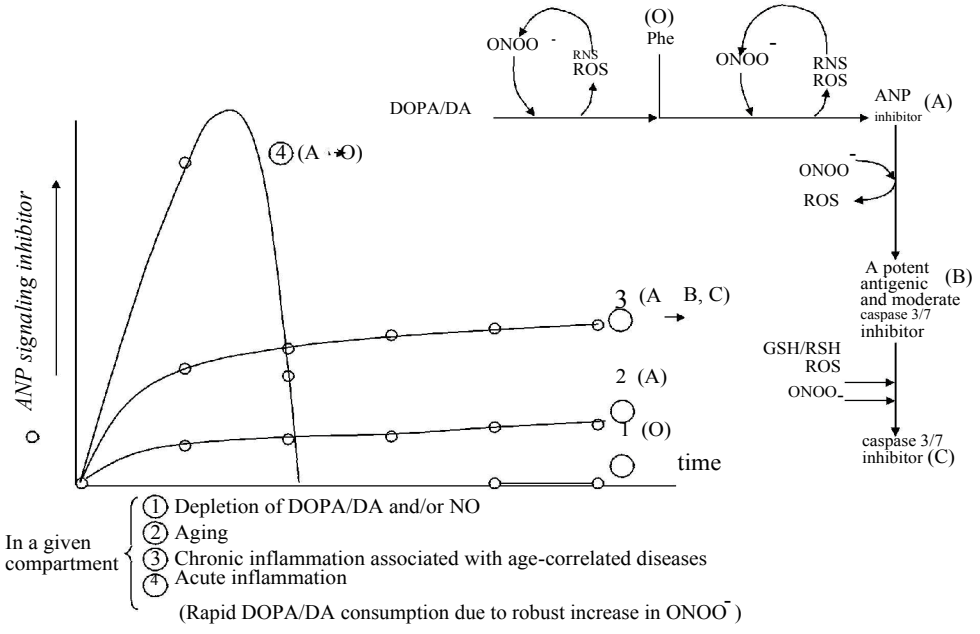

Figure 3: Formation of indole-2,3-dione (ANP binding/signaling inhibitor) and its derivatives causative of inflammatory disease states such as autoimmune or cancer.

death in most cases precede the damage within terminal regions accompanying normal aging?

(6) How can we explain the efficacy of dietary restriction in the retardation of mitochondrial respiratory function deterioration via the preservation of enzyme activity?

Eventually, in view of the differences in the physicochemical natures of electrophiles determining preferential reactivity towards proteins and towards DNA/RNA, the implication of non-specific toxic oxygen species in both mtDNA damage and enzyme protein modifications may require further examination. Organisms employ flavoenzymes and cytochromes both for the detoxification of xenobiotics or drugs in the microsome and for electron transport in respiration. Such a close relationship between detoxification and respiration may also be supported by both experimental and clinical studies demonstrating the association between GSH depletion and mitochondrial damage. Non-oxidative GSH consumption with electrophiles, which is known to result in prolonged depletion of intracellular GSH due to the severe requirement for de novo GSH synthesis, has been shown in hepatocytes to cause mitochondrial Ca^{2+} release and swelling [10] indicative of the formation of a pore that is permeable to high molecular mass solutes in the inner mitochondrial membrane, which is a critical stage in apoptosis. Mitochondrial damage due to depleted GSH has also been implicated as a key factor responsible for the manifestation of acquired immunodeficiency syndrome (AIDS) [11-13] as well as cognitive and

motor deficits ascribed to neuronal death within catecholaminergic terminal regions and dopamine defect, cardiomyopathy [14] and cancer. GSH depletion is able to modulate, and in most cases enhance, mutagenicity, carcinogenicity, genotoxicity, teratogenicity and cytotoxicity exerted by electrophilic compounds [15-20] via reactivity with nucleophilic cellular macromolecules. The primacy of electrophiles that deplete GSH in a non-oxidative manner, but not ROS generation, in inducing lipid peroxidation to drive apoptosis may also be supported by the observation that the over-expression of BCL-2 associated with elevated GSH, which is unable to suppress hydrogen peroxide generation via menadione, blocks subsequent lipid peroxidation apoptosis caused by this agent [21]. To advance and extend the hypothesis that GSH depletes electrophiles, it should also be noted that an important determinant of the specific cellular nucleophiles that are preferentially attacked by a given electrophilic compound is the physicochemical nature of the electrophilic center.

Based on the assumption that an increase in electrophiles for the consumption of GSH in a non-oxidative manner is responsible for the selective impairment of substrate (quinone) binding, the enzyme inhibition of complex I and the accumulation of mtDNA damage with age, it appears quite natural to consider the following possibilities for examination:

(1) Electrophiles exhibiting both soft and hard electrophilicity are the most detrimental due to their dual reactivities towards GSH/proteins and towards DNA/RNA.

(2) Under condition involving depleted GSH, a given electrophile undergoes an alternative substitution, which would not occur in the presence of an ample GSH supply, to acquire enhanced electrophilicity and/or other biological actions causative of pathological changes.

(3) Depleting GSH raises the possibility and/or the feasibility of an electrophile causing mtDNA damage and protein modifications as well as permitting the elicitation of specific pathophysiological actions by a given electrophile.

(4) GSH conjugated with a given electrophile exerts deleterious effects on nucleophiles through enzymatic or non-enzymatic degradation.

(5) Such electrophiles are produced solely at the expense of molecules exclusively depending on dietary intake.

Given that an increase in GSH-depleting electrophile(s) underlies a single molecular mechanism to explain how age-correlated diseases are increasing in incidence, the following points should be considered demands that must be met. In view of the selective vulnerability of catecholaminergic terminal regions in aging and age-correlated diseases, the formation of GSH-depleting electrophile(s) should be greatly stimulated in close proximity to catecholamine-producing cells with peak production being achieved within catecholaminergic terminal regions. The fundamental features common to age-correlated diseases include a profound dopamine defect [22-24] exceeding catecholamine-producing cell loss, suggesting that oxidative consumption of 3,4-dihydroxyphenylalanine (DOPA) and dopamine occurring in extra-neuronal spaces and non-neuronal cells precedes catecholaminergic cellular demise. Such extra-neuronally consumed DOPA/dopamine may represent a theoretical model that agrees with the observation of transient over activation of tyrosine hydroxylase (TH)-positive cells during early or developing stages of age-correlated diseases preceding a dopamine defect, the mechanism of which remains unrevealed. GSH-depleting electrophile(s) may be produced solely at the expense of DOPA and dopamine. Abnormalities in the determination of cell fate between cell death and growth are a predominant feature common to age-correlated diseases. The importance of the balance between protease activity and intrinsic protease inhibitors has long been appreciated with regard to the pathophysiology in which the regulation of cell fate is predominantly involved, as represented by angiogenesis. In this regard, the sulfhydryl requirement for protease activities has attracted much interest for decades. Paradoxical double roles for intracellular GSH, presumably dependent on quantity, in determining cell fate have been well-documented in terms of both apoptotic and proliferative pathways. These include the implication of GSH levels in the regulation of thiol protease-caspase 3 activities in HIV-1 [11,12,25,26], facilitating the assumption that sulfhydryl reactive-electrophiles may modulate progression towards death or survival via a mechanism involving competition between GSH and thiol proteases, -such as caspase 3, as targets for attack by electrophiles. The hypothesis of GSH-depleting electrophiles may be advanced to suggest that the production of electrophiles should depend on certain biochemical mechanisms involving ROS, NO, DOPA and catecholamines, considering the implications of distorted cell fate regulation in age-correlated diseases. The reasons for this hypothesis are explained as follows.

A close and inverse relationship between apoptotic and proliferative pathways has been revealed in cells of any developmental origin, from embryonic development through age-correlated disease states. During development, embryonic cell death occurs within zones of cell proliferation rather than regions containing postmitotic neurons [27]. Caspase 3 inhibition alone can promote cell proliferation by preventing the apoptotic pathway [28]. Anti-angiogenic agents cause cell death [29]. Angiogenic factors such as NO, reactive oxygen species (ROS) and DOPA/catecholamines [30] are also implicated in the apoptotic process [31-33]. In addition, the timing and sites of iNOS and TH in organogenesis [34-46] strongly suggest that both enzymes may contribute their downstream products, i.e., NO, ROS, DOPA and catecholamines, to the regulation of cell fate. This possibility is raised by accumulating evidence, described below, suggesting that a certain biochemical mechanism involving NO, ROS, DOPA and catecholamines operates a switch to induce the proliferation of non-neuronal cells in close proximity to the degeneration of catecholamine-producing cells. To go a step further, one assumption is that a distinct threshold to trigger a switch between cell death or growth depends on the absolute quantities of peroxynitrite (ONOO-) and DOPA/dopamine in a compartment. Important experiments examining cancer growth have revealed that ONOO- supplied by host cells quantitatively determines tumor growth and tumor-associated angiogenesis by inducing ONOO- resulting in iNOS in cancer cells [47]. In addition, NGF-induced NOS not only reverses the apoptotic effects of 6-hydroxydopamine (6-OHDA) in catecholamine-producing cells but also exerts potent angiogenic and proliferative actions on adjacent non-neuronal cells [48]. Caspase 3 activation and cell death induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can be reversed by NGF via the suppression of caspase 3 activity [49]. It has also been demonstrated that pharmacological blockade or genetic knockout of nNOS activity prevents methamphetamine (METH)-induced toxicity [50]. ONOO--mediated nitration of free and protein-bound tyrosine in catecholamine terminal regions has been described not only in MPTP-treated animals [51] but also aged mammals [52]. These observations lead to the advanced hypothesis that in the presence of DOPA or catecholamines, the absolute quantity of ONOO- determines the formation rate of diffusible

electrophiles that trigger a switch to apoptosis or, if modified in the reaction with ONOO-, reverse apoptosis by inhibiting caspase 3 and elicit the promotion of mesodermal/mesenchymal cell proliferation. Such further modification by ONOO-, although exemplified under pharmacological conditions in in vitro studies employing ROS-generating chemicals, may not occur in neuronal cells in vivo. The implication of DOPA or dopamine quinones and related metabolites as endogenous cytotoxic electrophiles [53] derived from the reaction between DOPA or catecholamines and ONOO- [54] in age-correlated diseases has also been discussed in the context of the cytoprotective roles of glutathione S-transferases (GSTs) against intracellular DOPA or dopamine non-oxidative cytotoxicity [33]. Up-regulated GST isoforms, indicative of local increases in GSH-depleting lipophiles with some degree of electrophilicity [55,56] on the carbon attacked, have been observed in diseased regions during age-correlated diseases and are suggested to exert cytoprotective actions, at least in part, by catalyzing the conjugation of GSH with DOPA or dopamine quinones [33-57]. However, physicochemical nature of DOPA- and dopamine-derived quinones may limit appreciation of the pathogenic roles of these quinones in age-correlated diseases that involve DNA/RNA damage, protein modifications, functional deterioration of enzymes and concomitant alteration of GSH status.

The ability of compounds to deplete GSH depends on whether they are good substrates for GSTs and/or rapid GSH conjugate formation preceding ROS-mediated oxidized GSH (GSSG) formation. In addition, the cytotoxicity of GSH-conjugated electrophiles is assumed to be exerted instead via increasing electrophilicity on the carbon attacked or the preservation of active carbonyl rather than via ROS-generating redox cycling [58-63]. In this regard, non topa, p-quinone of topa 6-OHDA, p-quinone of 6-OHDA, dopaquinone, and dopamine o-quinone [53] may not be expected to be strong candidates to cause GSH depletion associated with cellular macromolecule damage. Quinones that exert cytotoxicity via ROS-generating redox cycling rather than via non-oxidative GSH consumption are well-known to induce increased GSH levels as a consequence of an adaptive response to transiently depressed GSH levels. Norepinephrine completely neutralizes 6-OHDA and topa as cytotoxic agents,both of which are known to kill cells through ROS production. The weak sulfhydryl reactivities of p-quinones of topa and 6-OHDA have been confirmed [53]. In general, for dopaquinones and dopamine quinones, GSH conjugation is assumed neither to reduce the ability of quinone to undergo redox cycling or its ability to maintain electrophilicity of the carbon on the 6-membered ring, as both a relatively stable benzene ring formed during conjugation and the chemical characteristics of GSH, which possesses high nucleophilicity, are properties that result in overwhelmingly poor electron donation. Support for this view also comes from previous observations that GSH conjugates of quinones undergo redox cycling in some cases more rapidly than the parent quinones [64,65]. Elicitation of complete quinone detoxification depends on the electrophile-responsive induction of DT-diaphorase, glucuronosyl transferases, and sulfontransferases rather than GST-mediated elimination into extracellular spaces [63]. Specific up-regulation of GSTs may reflect an adaptive response to increased electrophiles that resist the activity of an export pump, which would preferentially eliminate GSH-conjugates in favor of GSH itself, would possess electrophilicity maintained beyond GSH conjugation and/or would distort cellular detoxification machinery. Quinones serve as substrates for flavoenzymes, including NADPH-cytochromeP_{450}-reductase, DT-diaphorase, NADPH-cytochrome b_5 reductase and NADH ubiquinone oxidoreductases, stimulating NAD(P)H oxidation [66].

Quinones appear unlikely to cause either a severe decline in NAD^+-linked oxidation in aging to a greater extent than Complex I or profound GSH depletion. Thus, the search for a biologically active electrophile that steadily increases with age and is produced at the expense of DOPA/dopamine depends on the quantity of ONOO-both for synthesis and further modification, the non-oxidative depletion of GSH as a good substrate for GSTs such as α,β-unsaturated carbonyl compounds, the preservation of active carbonyl group(s) or the maintenance of electrophilicity on the carbon while undergoing conjugation with GSH, and the possession of reactivities towards both proteins and DNA/RNA, causing the functional deterioration of $NAD(P)^+$-dependent oxidoreductases; such an electrophile, would attract much interest in the pursuit of a common molecular basis underlying age-correlated diseases. Indole-2,3-dione is a biologically active electrophile produced in every fundamental cycle (Figure 1), possessing the ability to interfere with electron-borne information via a complex mechanism, most notably by inhibiting the actions of either ATP and NAD(P)H towards ANP receptors [67], peripheral benzodiazepine receptors(PBR) [68], and $NAD(P)^+$- utilizing oxidoreductases [69], as discussed below. Indole-2,3-dione also inhibits the majority of detoxifying enzymes including MAO [70], [71] and phosphatases [72], suggesting that it affects not only detoxication but also metabolism of purines, pterins and neurotransmitters. Of particular interest is the fact that PBR and MAO B, both of which are the targets of indole-2,3-dione inhibitory actions localize to outer/inner mitochondrial membranes. Among various plants and bacteria, indole-2,3-dione has long been identified for metabolic pathways with distinctive enzymes, which have been lost in animals. With a bright orange color indicative of blue light absorption, indole-2,3-dione which interferes with $NAD(P)^+$- utilizing flavoenzymes may affect circadian light input pathways. Its chemical structure exhibits the similarity to adenine and may facilitate competition or replacement with ATP and NAD(P)H for this molecule, which in concert with short wavelength light absorption, could result in the disruption of synchronization between these enzyme functions and the environment via exogenous stimuli. Indole-2,3-dione is endogenously synthesized at the expense of the essential amino acid phenylalanine (Phe) [69,70] in the presence of DOPA/dopamine-derived quinones and ONOO- [70]. Of great importance is the fact that 2 ONOO- are required for the formation of 1 indole-2,3-dione, suggesting the existence of a distinct threshold for the absolute quantity of ONOO- with regard to indole-2,3-dione formation in a given compartment.

A rapid increase in indole-2,3-dione formation of up to 10 folds [73] also supports the involvement of a free radical-mediated synthesis process, although the participation of L-aromatic amino acid decarboxylases and/or MAO catalytic activity cannot be ruled out in view of its chemical structure. This sequential reaction process appears quite similar to that observed in lucigenin-based chemiluminescence [74], in which a steady increase in chemiluminescence levels continues in the presence of re-generated ROS until lucigenin itself is completely consumed and decayed to its end products. The photoemission arising from a lucigenin-based chemiluminescence system is distinguishable from that arising from other systems, including luciferin derivative-based systems because the former is driven by the energy drop during the oxidative decay of lucigenin and therefore does not directly quantify superoxide anions or singlet oxygens, although it appears to roughly correlate with the rate of ROS generation within a certain window. In addition, photoemission that roughly reflects the decay rate of lucigenin is discontinuously and dramatically increased whenever the rate of

ROS generation in a compartment exceeds a putative threshold, indicating the appropriateness of the 'phase transition' theory (Figure 4). A drastic increase in photoemission that is always followed by a rapid fall to below the basal levels or the nadir indicates that the rapid degradation of a 'parent' lucigenin due to the ROS supply exceeding a threshold terminates the sequential reaction. A similar phenomenon has been observed for indole-2,3-dione formation. The results shown in Figure 4 suggest that as long as the ROS generation rate never exceeds a putative threshold towards 'phase transition' in the lucigenin degradation reaction, the photoemission arising from the lucigenin system approximately reflects the rate of ROS generation from XO, which is correlated with the ROS-mediated degradation of lucigenin but is never precisely indicative of ROS levels themselves.

The net outcome of sequential reactions towards indole-2,3-dione formation never decreases reactive nitrogen species (RNS), ROS or quinones in a compartment (Figure 3) but rather promotes DOPA/dopamine oxidative consumption, resulting in dopamine defects if such reactions occur in extra-neuronal spaces and non-neuronal cells. Presuming that physiologically supplied Phe is not a limiting factor, the formation rate of indole-2,3-dione exhibits a steady state or a steady increase under normal physiological conditions, with a rapid fall only accomplished via the depletion of either DOPA or NO [69,73,75]. Given the similarity to lucigenin-derived photoemission, the formation rate of indole-2,3-dione increases discontinuously whenever the rate of ONOO- generation in a compartment exceeds a putative threshold. Despite being diffusible and permeable, indole-2,3-dione exhibits a distinctive distribution in mammalian blood, tissues and organs at concentrations comparable to those of catecholamines, with higher concentrations in the supra-cervical ganglion (~3.8 mM: based on the direct proportion of MAO inhibitory activity to indole-2,3-dione), vas deferens (~75 μM), seminal vesicle (~30 μM), kidney (~1.5 μM), lung(~0.8 μM), hippocampus (~0.9 μM), striatum (~0.8 μM) and cerebellum (~0.9 μM) [68,76], where nNOS is strongly expressed and colocalized with TH-positive cells. In view of the selective vulnerability of the hippocampus or striatum in age-correlated apoptosis and the selective survival of the vas deferens and seminal vesicles during the developmental degeneration of the mesonephros, indole-2,3-dione formation may be closely related to a switch between apoptotic cell death and growth from embryogenesis through aging. This notion is compatible with the assumption inferred from the previous observation that a several-fold increase in indole-2,3-dione is elicited by specific agonists to 5-HT 2A/2C receptors [75], which have long been known to mediate the potentiation of NOS and TH activities and have recently been appreciated as contributors to the ontogenetic determination of cell fate [77,78]. The unique physicochemical nature of indole-2,3-dione supports the view that this molecule is a strong candidate for GSH depletion simultaneously causing mtDNA damage and protein modifications as well as triggering a switch for cell death or growth. It is a heterocyclic quinonoid harboring a benzene ring as a soft electrophile and a 5-membered ring with active carbonyl groups as a hard electrophile, which exhibit preferences towards proteins and DNA/RNA, respectively. The local indole-2,3-dione concentration is thought to be the result of a dynamic equilibrium between its synthesis, GSH-conjugate-mediated metabolism and other modifications such as nitration. This view is compatible with the inverse relationship between diurnal variations in GSH and indole-2,3-dione in human blood(data not shown). As a soft electrophile, indole-2,3-dione undergoes GST-catalyzed conjugation with GSH at the 4 or 6 position under conditions involving an ample GSH supply due to its appropriateness as a good substrate for GSTs. The resonance canonical form can also participate, suggesting that the electrophilicity on the carbon at the 3 position may not be high enough to attack DNA/RNA because of the electro-withdrawing properties of the glutathionyl group for the nucleophilic compound. These GSH conjugates are eliminated into extracellular fluids, preferentially to GSH itself or other molecules, particularly under conditions involving increased ONOO-production. The 6-glutathionyl conjugate can undergo nucleophilic displacement with a nitro group via ONOO- attack and subsequently undergo nucleophilic attack by proteins but not by DNA/RNA due to the comparative softness of its electrophilicity (Figure 5). However, in cases where ONOO- attack on indole-2,3-dione precedes other modifications, the consequences are quite different and more detrimental (Figure 5). Initial attack by ONOO- is expected to occur at the 5 position, but not at positions 4 or 6, to form 5-nitro substituted indole-2,3-dione possessing significantly enhanced electrophilicity on the carbon at position 3; this 5-nitro substituted indole-2,3-dione would attack DNA/RNA as a hard electrophile or otherwise undergo nucleophilic displacement with GSH or proteins to form sulfide, which is oxidized by microsomal flavin-containing monooxygenase towards sulfone. The former case could result in the liberation of carbon monoxide(CO) in contact

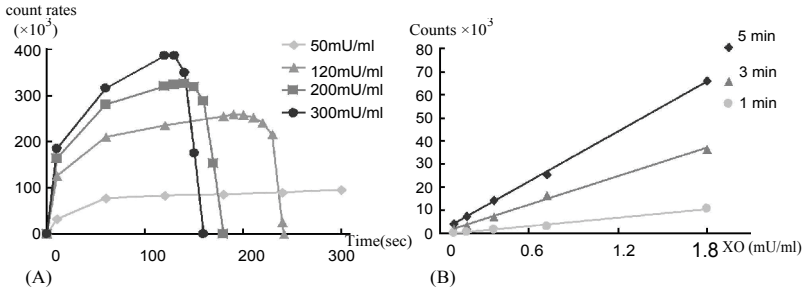

Figure 4: (a) Lucigenin-based chemiluminescence in hypoxanthine-XO ROS generating system. Lucigenin (0.250 mM) is employed as the electron acceptor. The reaction was started by the simultaneous addition of hypoxanthine and lucigenin. Photoemission was measured every second for 5 minutes in a luminometer (model 301, Aloka). A buffer blank was subtracted from each reading before transformation of the data. To examine whether lucigenin-dependent luminescence represents the rate of ROS-mediated degradation of lucigenin itself, rather than the generation of ROS directly deriving from XO activity, XO activity over a range from 50 to 300 mU is applied. According to the rough calculation, 110 mU of XO can every minute provide ROS exceeding the required for rapid degradation of all the lucigenin applied.

(b) Lucigenin-dependent luminescence in the XO-hypoxanthine system at XO concentrations as low as 0.1-2 mU. Each solid line indicates the counts integrated over 1, 3, 5 minutes respectively in a linear function of XO concentration over a range from 0.1-2 mU of XO, corresponding to 10^{-10}–2 x 10^{-9} M superoxide anion generation.

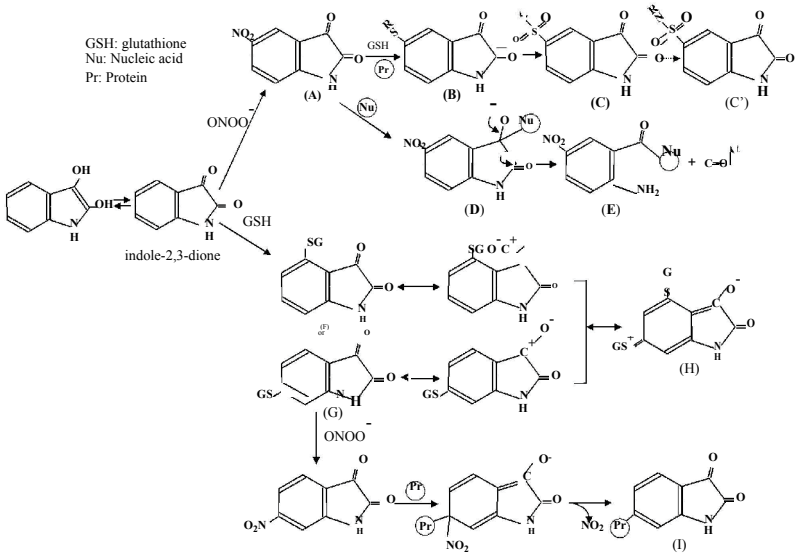

Figure 5: Schematic drawing of indole-2,3-dione and its derivatives, shown with redox cycling.

with DNA/RNA, whereas the latter case could eventually bring about the generation of sulfonamide of indole-2,3-dione, which are potent caspase 3 and 7 inhibitors [28], although detailed reaction processes require further investigation. CO liberation from a indole-2,3-dione derivative covalently bound to DNA/RNA is thought to functionally deteriorate cytochromes because indole-2,3-dione has long been known to target MAO and PBR on the inner/outer mitochondrial membranes.

Given that the capability of the heterocyclic quinonoid, indole-2,3-dione to undergo redox cycling is much less than that of catechol quinones with 6-membered rings stabilized by benzene ring formation, indole-2,3-dione may consume local GSH as a soft electrophile almost exclusively in a non-oxidative manner. Prolonged GSH depletion raises the potential for an initial attack on this quinonoid by ONOO- towards the generation of CO or a caspase inhibitor as well as accessibility to nucleophilic macromolecule conjugates without a loss of electrophilicity on the carbon. Support for indole-2,3-dione and its derivatives as contributing factors in age-correlated diseases also derives from both their biological actions and observations of specific increases in indole-2,3-dione up to 8-10-fold during stress [73] as well as in cancer [79], vasculopathy-based diseases [80] and neurodegenerative diseases, with levels that correlate with severity [81]. The close relationship between indole-2,3-dione and the stress response is supported by distinct seasonal variations in rats demonstrating an inverse correlation between its production with the open-air temperature. The most potent physiological actions include the inhibition of ANP receptor binding, G-protein mediated signaling, NAD(P)H-oxidizing flavoenzymes, peripheral benzodiazepine receptor(PBR) binding, and glucose transport [82], permitting the assumption that indole-2,3-dione may modulate local inflammation, vasomotor tone, extracellular fluid volume and content, local steroidogenesis and the metabolism of cholesterol and glucose, both directly and indirectly by discouraging the benign actions of PBR and ANP in terms of the suppression of TH and iNOS activation/induction. As mentioned above, the 5-nitro derivative has been shown to be a moderate caspase 3 inhibitor with some antigenic properties, whereas further nucleophilic displacement with sulfhydryls at 5 the position may give rise to one of the most potent caspase 3 inhibitors, sulfonamide of indole-2,3-dione. Indole-2,3-dione inhibits the majority of detoxifying enzymes, including MAO B, esterases, XO and phosphatases. In view of the extremely high concentrations of indole-2,3-dione in close proximity to catecholamine-producing cells [68], it may be assumed that, in some cases, indole-2,3-dione may resist enzymatic detoxification and also confer increased resistance upon electrophiles, including catechol quinines, to cellular detoxification machinery.

It is worthwhile to hypothesize that

(1) Age-correlated increases in indole-2,3-dione, which is an endogenous inhibitor of both ANP binding/signaling and PBR binding/signaling, followed by adaptive increases in plasma ANP represents a molecular mechanism of aging.

(2) This is based on the assumption that

(a) The formation rate of indole-2,3-dione is determined by the absolute quantity of both ONOO- and DOPA/dopamine in a given compartment as long as the Phe supply is not a limiting factor.

(b) The rate of 5-nitro substitution for indole-2,3-dione is determined by the absolute quantities of both indole-2,3-dione and ONOO- in a given compartment if local GSH levels never exceed a putative threshold. Investigations will determine whether the age-correlated increase in indole-2,3-dione represents a proper aging mechanism that meets the above-mentioned demands of the proposed theory.

Results

Physicochemical nature and various derivatives of indole-2,3-dione (Figure 5).

Discussion

Considering the unique physiological nature and distinct biological actions of indole-2,3-dione and its derivatives, the data presented in this report appear to be in appreciable agreement with the theoretical demands for a model to explain aging mechanisms based on the close link between detoxification, respiration, biosynthesis and the stress

response, with particular primacy given to the impaired detoxification of electrophiles [83]. Cancer, vasculopathy-based diseases and neurodegenerative diseases have also been designated age-correlated disease states given that their incidence increases with age. They must share a molecular mechanism that aligns with a single mechanism of aging. In view of the selective vulnerability of catecholaminergic cells associated with the induced expression of stress proteins and markers of inflammation in cells of mesodermal/mesenchymal origin within catecholaminergic terminal regions, a number of studies have investigated the pathological relevance of DOPA, catecholamines, ROS and RNS in age-correlated diseases. The important questions that remain involve the profound dopamine defect preceding and exceeding catecholamine-producing cell demise and its association with functional disruption of mitochondrial complex I throughout the body. Apart from diet, dopamine exclusively originates in DOPA produced by catecholamine-producing cells throughout life with the exception of the early embryo, which depends on the maternal supply of DOPA, up to approximately E8.5, when primordial TH-positive cells appear. However, dopamine levels far exceed those of norepinephrine and depend on the supply from the non-neuronal cells, which are the major sites of dopamine synthesis [84].

The first working hypothesis of this study is that a profound dopamine defect should be accounted for by the steady increase in DOPA/dopamine oxidative consumption that occurs also in extraneuronal spaces and non-neuronal cells with age, rather than nitration-mediated deterioration of TH activity. Based on the assumption that diffusible molecules form only at the expense of local DOPA/dopamine in the presence of ONOO- and operate a switch to flip between cell death and growth, the physiochemical nature of electrophilic center, a possible modification induced by increased ONOO- and subsequent nucleophilic displacement and its influence on detoxifying enzyme activity for the candidate electrophile indole-2,3-dione are highlighted in this study with particular emphasis on the relationship between GSH status and mtDNA damage. The strict DOPA/dopamine requirement for indole-2,3-dione formation has already been confirmed [69,73,75]. Support for the first working hypothesis also comes from accumulating evidence in experimental models employing DOPA, dopamine and chemically divergent neurotoxins in catecholamine-producing cells, such as METH, MPTP, 6-OHDA and dieldrin. First, the evidence that chemically divergent compounds induce a ROS- and caspase3 activation-mediated common apoptotic pathway culminating in the selective cell death of catecholamine-producing cells [85] indicates the involvement of DOPA or catecholamine oxidation in a common molecular mechanism underlying the selective vulnerability of catecholamine-producing cells. The fact that the inhibition of tyrosine hydroxylase alone suppresses dieldrin-induced ROS generation and DNA fragmentation [86] further confirms the participation of DOPA or catecholamines in the apoptotic pathway and suggests that the augmentation of ROS is caused by the oxidation of DOPA or catecholamines. Selective catecholaminergic cell death following microglial proliferation associated with increases in HSP [70] expression and inflammatory markers, such as the induction of iNOS, which is widely observed within catecholaminergic terminal regions in METH-treated animals [87] suggests the possibility that diffusible small molecule(s) that form extra-neuronally at the expense of DOPA/catecholamines in the reaction with ONOO- may be responsible for neuronal death and non-neuronal proliferation in association with a profound dopamine defect.

The second working hypothesis in this study is as follows: the paradoxical double roles for ONOO- in neuronal cell death and non-neuronal cell proliferation can be tested, given that ONOO- participates in both the formation of a neurotoxic electrophile and its modification in non-neuronal cells, leading to the generation of a potent anti-apoptotic electrophile. With regard to both the formation of the above-mentioned electrophile at the expense of DOPA/dopamine and its subsequent modification, distinct thresholds for the absolute quantity of ONOO- in a given compartment should be expected. The first threshold originates from the requirement of 2 ONOO- molecules for the formation of 1 electrophile, as mentioned previously, and the second threshold originates from the presumable competition with nucleophilic attack by cellular sulfhydryls. The existence of distinct thresholds for ONOO- that are common to the apoptotic and proliferative pathways enables the synchronized operation of a switch towards neuronal cell death and towards non-neuronal cell proliferation, as well as enabling the age-correlated manifestations of specific pathological conditions that are distinguishable from each other, such as cancer, vasculopathy and neurodegeneration; these conditions may be distinguished quantitatively, although not qualitatively, from normal aging and embryonic development because the sequence of events depends on NO and DOPA/dopamine alone. In this regard, the great similarities between indole-2,3-dione-generating processes and lucigenin-based chemiluminescence as well as the strict requirement for ONOO- -mediated 5-nitro substitution of indole-2,3-dione for either the generation of a potent caspase 3 inihibitor [28] or CO liberation in contact with DNA/RNA may support this electrophile as a strong candidate for triggering a switch to neuronwardsal cell death, which is associated with non-neuronal cell proliferation. Because the conversion of sulfide of indole-2,3-dione via sulfone to sulfonamide of indole-2,3-dione, the caspase 3 inhibitor, may require further ONOO- action following flavin-containing monooxygenase activity, the degree of ONOO- required for the generation of the caspase 3 inhibitor appears much greater than that required for the formation of a covalent bond to DNA/RNA.

The fact that the synthesis of the potent caspase 3 inhibitor would not occur without 5-nitro substitution by ONOO- may account for the dependency of carcinogenesis and other pathological angiogeneses on the absolute quantity of ONOO-. ANP binding/signaling inhibition by indole-2,3-dione may also promote carcinogenesis and angiogenesis by inhibiting ANP actions that suppress both VEGF synthesis and release. That ONOO- attack following GSH conjugation at position 6 can result in protein modifications, but neither DNA/RNA damage nor caspase inhibition suggests that intrinsic GSH levels are critically implicated in the determination of cell fate, particularly under conditions involving increased ONOO-. The third working hypothesis is that the controversial problems underlying mitochondrial damages in aging are not ascribable to oxidative stress by known oxygen toxic species. These include the discrepancy between decreased rotenone sensitivity for complex I throughout all cell types in aging and the specific lack of decreased electron transfer in aged platelet mitochondria [88]. Based on the theoretical model involving a GSH-depleting electrophile, this might be accounted for by the potential lag time between interference of the GSH-depleting electrophile with the binding site of the acceptor substrate (quinine) and induced mtDNA damage, originating from differences in probabilities and/or accessibilities. All unique physicochemical properties of the endogenous quinonoid indole-2,3-dione appear to support the view that this electrophile is a strong candidate. Indole-2,3-dione itself may cause a decline in NAD+-linked oxidation because it appears to withdraw electrons from NAD(P)H at the NAD(P)+ binding sites on oxidases. Increasing ONOO-

production with age would result in nucleophilic substitution with a nitro group at the 6 position following GSH conjugation, leading to enzyme-induced protein modifications. Non-oxidative GSH depletion precipitated by indole-2,3-dione may allow the preceding ONOO- attack, leading to the liberation of cytochrome-inhibiting CO and concomitant covalent binding to DNA/RNA, as well as the predisposition of mitochondria to oxidative stress. Although it has not been determined what promotes the process towards the generation of sulfonamide of indole-2,3-dione, the potent caspase 3 inhibitor, both flavin-containing monooxygenase activity [1] and strong oxidizing compounds at least are required. The interference with blue light reception by indole-2,3-dione may rise in importance particularly in terms of the variability in the functional deterioration of respiratory enzyme activities in aging. Indole-2,3-dione may affect the ability of blue light receptor enzymes to attract electrons from NAD(P)H to a greater extent in oxidases exhibiting some coincidence of absorbance maximum.

In general FAD-bound flavoenzymes exhibit absorbance maximum with a range of 380-450 nm, which is lost when electrons are withdrawn from the substrate NAD(P)H. The circadian-rhythmic flavoenzyme XO, which is capable of withdrawing electrons from NAD(P)H, participates in blue light input pathways [83] and is blocked by allopurinol, the inhibitor for both XO and XDH, which lacks circadian rhythmicity. XO inhibition by indole-2,3-dione may be associated with the blockade of blue light reception presumably via preferential withdrawal of electrons from substrates including NAD(P)H. Of interest is that the inhibition of NAD(P)H oxidase by indole-2,3-dione in human endothelial cells and smooth muscle cells does not occur in a cell-free system supplemented with ample NAD(P)H (data not shown), suggesting that indole-2,3-dione behaves competitively with NAD(P)H at sites of electron transfer from NAD(P)H. The fourth working hypothesis is that most manifestations both in normal aging and in age-correlated diseases, are merely secondary effects derived from the intrinsic mechanism of aging. Stress responses initially arising from oxygen consumption in ectodermal cells terminate with the neutralization of electron flow by sulfur-containing compounds produced primarily in endodermal cells through the detoxification (elimination) of electrophiles by detoxifying enzymes that originate in mesodermal/mesenchymal cells. The deterioration of the integrating role for ANP and glucocorticoid with age, which derived primarily from the mesodermal cells during the regulation of a dynamic equilibrium between electrophiles and nucleophilic sulfhydryls, causes altered outcomes of stress response in association with the disruption of electron-mediated transmission. This may be exemplified by the case of indole-2,3-dione, as mentioned below. Given the elicitation of inhibitory actions on G-protein-mediated signaling of ANP by indole-2,3-dione via interference with ATP binding sites [67] that are found in most conserved regions throughout ANPs, all members of the ANP family are presumably subject to indole-2,3-dione action. The extent of receptor binding inhibition may not differ significantly for varied types of receptors due to highly conserved extracellular regions [89]. Competitive inhibition of ANP binding by indole-2,3-dione should attract much interest with regard to the age-correlated manifestations originating from disrupted ANP actions associated with paradoxical elevation of plasma ANP levels. Because plasma ANP levels are determined simultaneously with degradation by ectoenzymes, the degree of clearance receptor binding and subsequent internalization, rather than the achievement of G-protein-mediated signalling [90], indole-2,3-dione may also be a contributing factor to the age-correlated increase in plasma ANP levels.

Hypertension and pathological angiogenesis may be attributable to disrupted ANP actions to suppress TH [91], iNOS [92], and vascular endothelial growth factor (VEGF) [93,94]] in concert with other biological actions of indole-2,3-dione derivatives.The similarity to vitamin K [95] in terms of its chemical structure may confer increasing importance upon this electrophile in pathologies associated with aging. Indoel-2,3-dione inhibits the majority of detoxifying enzymes, including XO and MAO. This may lead to a paradoxical dissociation between decreased activity and up-regulated protein expression as an adaptation. The discrepancy between the age-correlated increase in iNOS and nNOS expression in age-associated vulnerable regions in the CNS and the paradoxical decrease in DT-diaphorase activity [96] may be partially accounted for by interfering electrophiles such as indole-2,3-dione. Certain controversial observations of XO activation in relation to vasculopathy may be derived in part from interference by electrophile(s). GST inhibition combined with GSH depletion by indole-2,3-dione may cause apoptotic neuron death. In contrast, under conditions of robust ONOO- production in cells of mesodermal/mesenchymal origin, 5-nitro substitution results in indole-2,3-dione resistance to benign GSH conjugation at the 4 or 6 positions, leading to a gradual recovery of GSH. Interference with cytochromes and flavoenzymes by indole-2,3-dione may result in the deterioration of transport and the metabolism of cholesterol and glucose, which, in concert with inhibition of PBR binding and glucose transport, may permit the manifestation of impaired steroidogenesis and insulin resistance in senescence. After birth, NOS and TH are both considered links between neural activity and activity-dependent regional vasomotor regulation.

However, during early embryonic development, the timing and the sites of transient but strong expression of iNOS and the primordial form of TH strongly support the pivotal roles of these enzymes in determining cell death or survival in organogenesis associated with robust vascularization. During the same developmental stage, oxygen tension dramatically increases, reflecting the rapid establishment of O_2 supply to the cardiovascular system. The view point emphasizing the critical roles of GSH and strongly expressed GSTs [97] at the same developmental stage in cytoprotection against electrophilic attack rather than oxidative stress may grow in importance, particularly with respect to the implication of GSH status in the determination of cell death, proliferation, transformation and migration, which, if distorted, would lead to teratogenesis, malformation or lethality. Although the biological actions of multifunctional ANP peptides encompass the regulation of extracellular fluid volume and content and acute and chronic vasomotor tone, the timing and sites of the developmental expression of ANP strongly suggest that ANP also participates in the determination of cell death and growth during organogenesis associated with rapid oxygenation in particular via the modulation of a certain signal from iNOS-expressing cells [98]. Additionaly, a close but mutually exclusive relationship between ANP and TH has also been supported by anatomical evidence during embryonic development [99-105], in particular with regard to the establishment of a mature heart rate and conducting system, as well as genetic targeting studies centered on hypoxia-inducible transcription factors [106] and the localization of ANP and its receptors in the adult nervous system [107]. The ANP gene, which harbors both AP1 and CRE within its promoter region [108] and is quite similar to the TH gene, permits the assumption that ANP responds to GSH-depleting electrophiles and synergistically to TH, modulates cell fate or the cell cycle and maintains a dynamic equilibrium between both local oxygen tension and oxygen-containing electrophiles. The regulatory role for ANP in determining blood pressure, peripheral resistance

or sodium/water balance may be merely a secondary effect derived from the intrinsic role of ANP mentioned above. PBR, as a mediator of the acute stimulation of steroidogenesis by hormones, constitutes one of the two cholesterol transport mechanisms. Predominantly localized on the outer/inner mitochondrial membrane contact sites in steroidogenic cells of the gonads, adrenal, placenta and brain, PBR functions as a mitochondrial cholesterol channel, modulates local and circulating glucocorticoid levels and in some cases promotes cell proliferation. PBR, induced at sites of inflammation, may also be involved in the balance between electrophiles and cellular sulfhydryls. Indole-2,3-dione may be formed at the expense of maternal DOPA in the presence of ONOO- derived from iNOS during early embryogenesis [109]. At the beginning of catecholamine production, which is intrinsic to the embryo from E8.5 towards E14, when peak expression is achieved and is associated with the establishment of the cardiovascular system and ANP expression, the DOPA supply switches from the maternal source to the primordial TH of the embryo itself. Thereafter, indole-2,3-dione is produced with each fundamental cycle and causes the deterioration of glucose transport, ANP action and glucocorticoid generation via ANP-or PBR-dependent mechanisms and CO-mediated cytochrome inhibition, which in turn concertedly disrupts the intrinsic mechanism to recover GSH depleted by indole-2,3-dione itself. This sequence of events should be paralleled with the functional deterioration of cytochromes and flavoenzymes with age. Disrupted complex I may reflect such concerted events. The electron reduction potential evoked by the stress-induced combustion of bi-radical oxygen is transformed into electrophilicity on adjacent atom(s), such as carbons, which in turn convey, transform or augment the information via the AP-1-mediated induction of proteins.

Conversely, electron reduction potential may be completely neutralized or detoxified by intrinsic sulfur displaying a similarity to the electron configuration to oxygen. Such an energy flow in the form of electron-mediated information from the ectodermal cells in the nervous system to the endodermal cells in the liver cells may be manipulated by glucocorticoids produced in the mesodermal/mesenchymal cells. Proper transmission of electron-mediated information beyond cell membranes is indispensable for life. However, in cases where electrophiles originating from oxygen consumption are not detoxified by intrinsic sulfur, the stress response would never terminate at the original equilibrium. The crisis caused by the imbalance between electrophiles and intrinsic sulfur is well characterized from as early as the first cleavage divisions of the embryo by the well-known phenomenon 2 cell block, suggesting that disrupted transmission of electron-mediated information caused by impaired detoxification of electrophiles may affect the determination of cell death and growth. The life spans of organisms may be limited not by aging of individual cells but by the development of distortions in operation of switch between cell death and growth with age, which should originate in the impaired detoxification of electrophiles that endure time and distance much better than free radicals that bear short-lived information in the form of unpaired electrons on the outer orbitals. A fundamental feature common to age-correlated diseases is the distorted determination of cell fate between death and proliferation, which should not occur as a consequence of the long-term accumulation of mtDNA damage manifested solely in post-mitotic cells. A single molecular mechanism throughout life should underlie both embryonic development and aging. In higher organisms both NOS and TH mediate neural activities of the sympathetic and parasympathetic nerves after the establishment of the peripheral nervous system [110]. However, in early development, iNOS contributes to cell proliferation and apoptotic death, presumably in cooperation with subsequently induced TH, by bearing the information derived from electrons; this process may descend from lower organisms such as bacteria. According to the theory of the bacterial origin of mitochondria, mitochondria should be quite sensitive to electron-borne information mediated by electron-donating molecules or compounds bearing unpaired electron(s) such as NO. Animals, which are all predators, have evolved light receptor oxidoreductases adapted for detoxification (elimination) [1] that are also indispensable for digestion and diet excretion, rather than photosynthesis and the immobilization of inorganic compounds. Incomplete detoxification (elimination) of the electrophile indole-2,3-dione originating from the essential amino acid Phe in the diet as well as production dependent on NOS and TH activities, could result in a decline in the ability of blue light receptor oxidases to withdraw electrons from NAD(P)H as well as a decreae in the efficiency of ADP/ATP-linked reactions, even in mitochondria of bacterial origin. Incomplete detoxification may also affect circadian clocks because light input pathways in animals exclusively depend on blue light reception. This may be the only reason explaining life is always limited. The author declares that there are no competing interests.

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