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ORIGINAL INVESTIGATION

Demands of an aging mechanism theory

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The endogenous quinonoid indole-2,3-dione, which has a bright orange color because it absorbs blue light, undergoes circadian control and has long been identified among plants and bacteria to be produced by metabolic pathways that are catalyzed by enzymes and are lost in animals. Here, I report the age-associated increase in indole-2,3-dione, which interferes with ATP or NAD(P)H binding to atrial natriuretic peptide receptors, peripheral benzodiazepine receptors and blue light receptor flavoenzymes. Indole-2,3-dione has dual reactivity towards glutathione (GSH)/proteins and DNA/RNA as both a hard and soft electrophile, and its formation rate depends on tyrosine hydroxylase activity and may affect the cell fate, GSH status, $NAD(P)^+$-linked oxidation, functions of cytochromes and ADP/ATP-linked energy conservation, at least through caspase 3 inhibition or carbon monoxide liberation.

Key words: Aging, Endogenous quinonoid indole-2,3-dione, ATP, NAD(P)H

INTRODUCTION

Organisms are continuously challenged by numerous exogenous and endogenous stressors from embryogenesis to death. The succession of stress responses enabling recovery to equilibrium constitutes life. The overall regulatory mechanisms of the stress response in animals, which integrates the neuroendocrine and immune system, have been designated the sympathoadrenomedullary system and hypothalamohypophysial system. The stress response begins with a metabolic burst in the nervous system, which is energetically driven by robust oxygen consumption in mitochondria, allowing the generation of electrophiles and free radicals that mediate electron-borne information or are otherwise detoxified. Enzymatic detoxication of xenobiotics and nutritionally useless electrophiles is considered a primordial form of the stress response, descending from unicellular organisms that were capable of an enormous range of catabolic activity. However, animals have evolved detoxifying enzymes adapted for elimination with much less specificity for each compound, rather than the utilization of high specificity detoxifying enzymes, as in bacteria [1]. Organisms are equipped with a number of oxidoreductases that are highly conserved from phototrophs to humans and utilized throughout detoxication, metabolism, biosynthesis and respiration, as observed for enzymes containing flavins, pterins or cytochromes and employing, in many cases, free $NAD(P)^+$ in addition to protein-bound FAD as a mediator of electron-borne information. These enzymes are capable of absorbing blue light and are components of circadian light input pathways as well as being under clock control, suggesting that they constitute a primordial stress response to light. Thus, the activities of blue light receptor enzymes are indispensable for synchronization with the environment of circadian clocks, comprising a rhythm generator in response to specific signals (*zeitgeber)*. NAD(P)H, the ubiquitously occurring electron donor conserved from anaerobes to aerobes,

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is 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 of the redox state, intracellular pH and metabolism of nitrogen and sulfur, leading to subtle changes in the outcome of the stress response that would otherwise terminate at the original equilibrium. The sequence of events may be accompanied by a decline in respiratory function in mitochondria. The mechanism of aging may consist of a deterioration of the maintenance of equilibrium between the stress response and detoxication.

It is conceivable that sulfur-containing tripeptide glutathione (GSH) is a strong candidate representing a link between the stress response, detoxication and aging because it is consumed to confer increased resistance of the early embryo to a stressor as early as the 2-cell stage when increased production of heat shock protein (HSP)70 family is also manifested [2]. Furthermore, it detoxifies redox-cycling compounds and electrophiles that cause DNA damage and protein modification throughout life, and also metabolizes hydrogen peroxide as the only defense available, particularly in mitochondria. Diurnal variations in GSH are clearly observed in the nervous system and liver in close relation to the oxidative stress cycle [3-6], which strongly suggests a reciprocal relationship in the stress response between the liver, the main reservoir of the systemic GSH pool, and the nervous system, which includes the organ with the highest rate of oxygen uptake and lipidperoxidation. The stress-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, affects gene expression, cell fate and cycle and membrane transport, with the highest extent being achieved in the nervous system because it has the highest 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 the stress response. Diurnal variations in animals are also clearly 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 at points of application of light. Disrupted inputs should accompany altered outputs, including intracellular redox sates, pH, and the metabolism of nitrogen and sulfur. GSH status may reflect diurnal variations in electron-borne information underlying detoxication, biosynthesis and respiration.

If a proper theoretical model of the aging mechanism exists, the demands of theory should be as follows:

1) aging should be accounted for by a fundamental cycle that occurs in succession from embryogenesis through death because aging originates in embryogenesis and reflects merely an aspect of development (Fig. 1);
2) a fundamental cycle should be based on the concept of stress response and involve inflammatory process because age-correlated disease states are also stress-related and involve inflammation (Fig. 1);
3) the mechanism responsible for the increasing incidence of age-correlated disease states with age must be in line with a single mechanism of aging. Any manifestation at the initiation of age-related disease states should be in line with a single mechanism of aging (Fig. 2);
4) quantitative changes in one or more constituents of a fundamental cycle must directly or indirectly cause a qualitative change leading to any manifestation of age-correlated disease states. Thus, a distinct threshold in quantity should exist for a component of a fundamental cycle to form a biologically active substance causative of a qualitatively distinguishable state associated with age-correlated diseases (Fig. 3).

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

Important problems that remain unsolved may be as follows: (1) Why is overall NAD$^+$-linked oxidation more severely compromised during aging than Complex I activity? (2) From what is derived the distinct variability in the presence and the extent of functional deterioration of activities among respiratory enzymes in aged mitochondria? (3) Why does decreased rotenone sensitivity for complex I indicative of specific deterioration with age of the physiological quinone(substrate) binding precede the disruption of electron transfer? (4) How can the association of complex I disruption throughout the body in cells of any developmental origin with a selective vulnerability of catecholaminergic terminal regions both in the periphery and in the brain, such as the hippocampus, striatum and salivary glands, be rationalized in aged animals? (5) How does mitochondrial theory parallel a mechanism of age-correlated increase in susceptibility to diseases in which the presence and the extent of

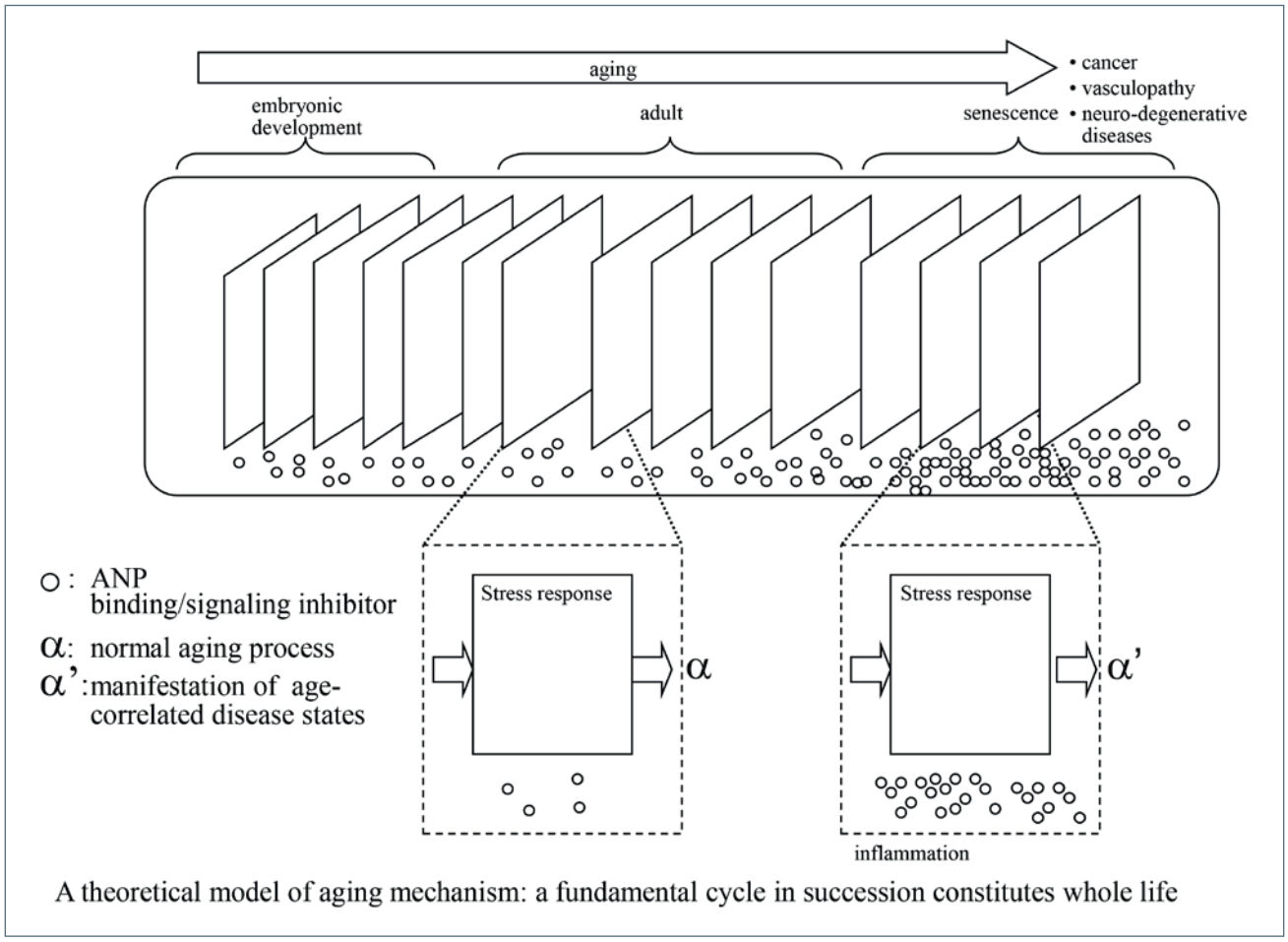

Figure 1. Theoretical model of the aging mechanism, employing a fundamental cycle. A fundamental cycle consisting of NO synthase and TH activities representing the stress response and constituting the whole life. The increase in the ANP binding/signaling inhibitor indole-2,3-dione is associated with inflammation.

a selective catecholaminergic cell death in most cases precedes that of damages within the terminal regions accompanying normal aging? (6) How could the efficacy of dietary restriction to retard the deterioration of mitochondrial respiratory function by preserving enzyme activity be explained?

Eventually, considering the differences in the physiochemical nature of electrophiles determining the preferential reactivity towards proteins and DNA/RNA, the implication of non-specific oxygen toxic species in both mtDNA damage and enzyme protein modifications may require further examination.

Organisms employ flavoenzymes and cytochromes both for detoxifying xenobiotics or drugs in the microsome and for transporting electrons during respiration. This close relationship between detoxication and respiration may also be supported by both experimental and clinical studies showing the association of GSH depletion with mitochondrial damage.

Non-oxidative GSH consumption with electrophiles, which is known to result in a 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 formation in the inner mitochondrial membrane of a pore that is permeable to high-molecular-mass solutes, representing a critical stage leading to apoptosis. Mitochondrial damage due to depleted GSH has also been implicated as a key factor that is responsible for the manifestations of acquired immunodeficiency syndrome (AIDS) [11-13], such as the cognitive and motor deficits ascribed to neuronal death within catecholaminergic terminal regions, dopamine defects, cardiomyopathy [14] and cancer. Depletion of GSH is able to modulate, and in most cases enhance, the mutagenicity, carcinogenicity, genotoxicity, teratogenicity and cytotoxicity exerted by electrophilic compounds [15-20] via reactivity with nucleophilic cellular macromolecules.

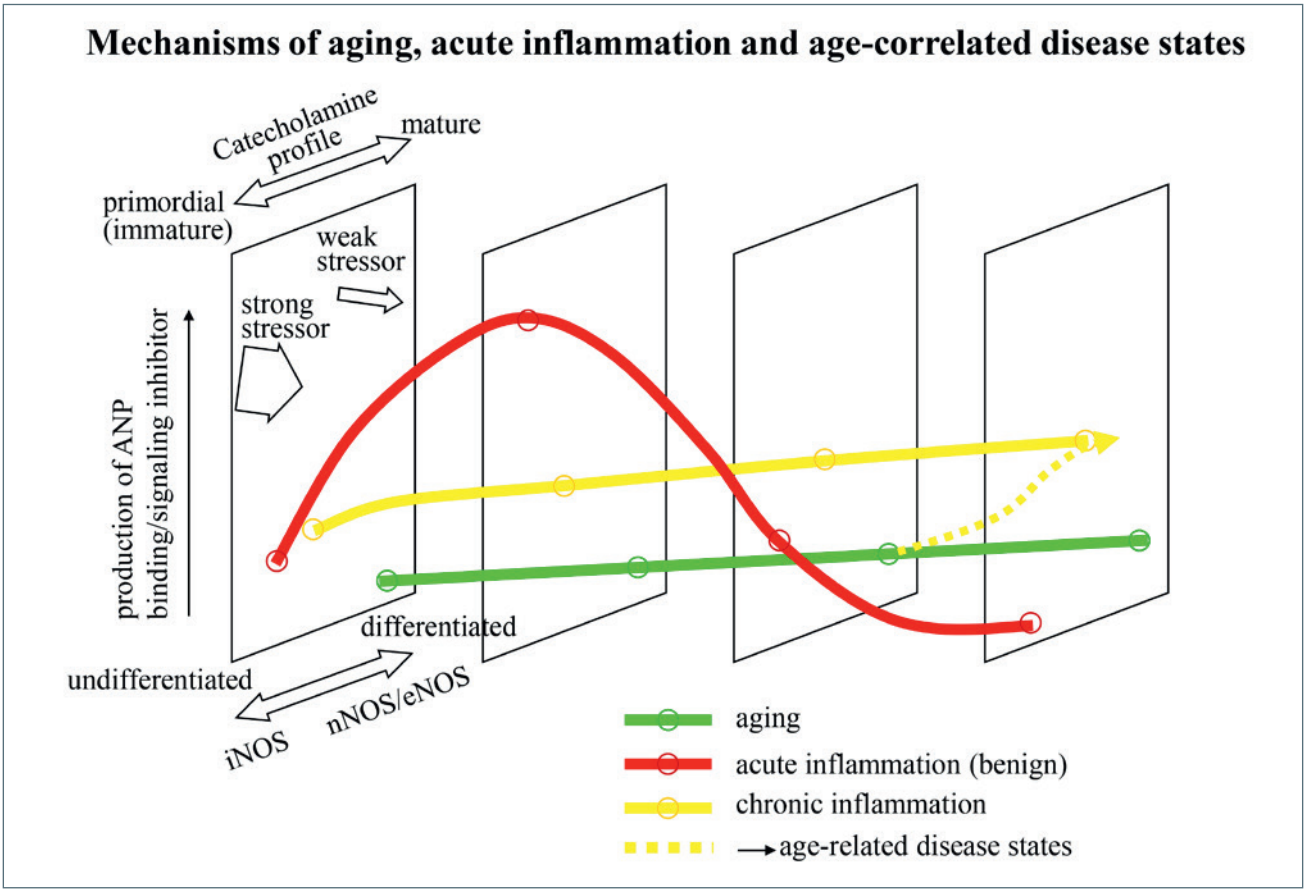

Figure 2. Schematic drawing of the patterns of formation of ANP binding/signaling inhibitor. The dotted line indicates how age-correlated diseases increase in incidence with age.

The primacy of electrophiles depletes GSH in a non-oxidative manner, but not ROS generation, to induce lipid peroxidation leading to apoptosis. This finding may also be supported by the observation that overexpression of BCL-2 associated with elevated GSH, which is unable to suppress hydrogen peroxide generation by menadione, blocks the subsequent lipid peroxidation apoptosis caused by this agent [21].

To advance and extend the hypothesis of GSH depletion of electrophiles, it should also be noted that an important determinant of the specific cellular nucleophiles preferentially attacked by a given electrophilic compound is the physiochemical nature of the electrophilic center.

Based on the assumption that an increase in electrophiles to consume GSH in a non-oxidative manner is responsible for a selective impairment of substrate (quinone) binding, enzyme inhibition of complex I and accumulating mtDNA damage with age, it seems quite natural to consider the following possibilities. (1) The electrophile displaying both soft and hard electrophilicity is most detrimental because of its dual reactivities towards GSH/proteins and towards DNA/RNA. (2) Under conditions of depleted GSH, a given electrophile undergoes an alternative substitution that would not occur in the presence of an ample GSH supply, to acquire enhanced electrophilicity and/or another biological action causative of a pathological change. (3) Depleting GSH raises the possibility and/or the accessibility for an electrophile to cause mtDNA damage and protein modifications and allows the elicitation of specific pathophysiological action by a given electrophile. (4) GSH conjugated a given electrophile exerts a deleterious effect on nucleophiles through enzymatic or non-enzymatic degradation. (5) Such electrophiles are produced solely at the expense of molecules that exclusively depend on dietary intake.

Given that the increase in GSH-depleting electrophile(s) underlies a single molecular mechanism by which age-correlated diseases are increasing in incidence with age, the following points are considered unmet demands.

Considering the selective vulnerability of catecholaminergic terminal regions in aging and age-correlated diseases, the formation of such GSH- depleting electrophile(s) should be greatly stimulated in close

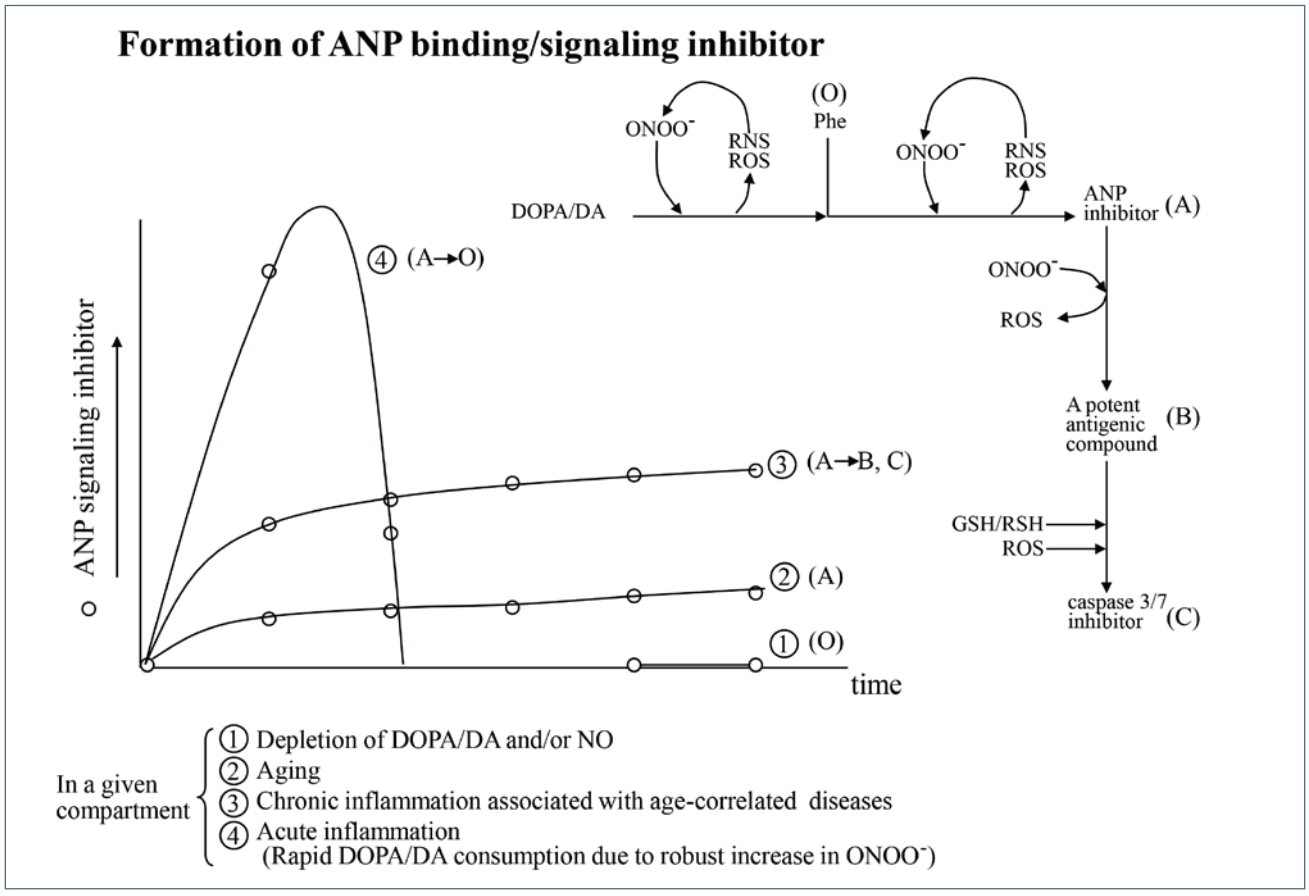

Figure 3. Formation of indole-2,3-dione (ANP binding/signaling inhibitor) and its derivatives, which cause inflammatory disease states, such as autoimmune conditions or cancer.

proximity to catecholamine-producing cells, with peak production being accomplished within catecholaminergic terminal regions.

The fundamental feature common to age-correlated diseases includes a profound dopamine defect [22-24] exceeding catecholamine-producing cell loss, suggesting that oxidative consumption of 3,4-dihydroxyphenylalanine (DOPA) and dopamine in extra-neuronal spaces and non-neuronal cells precedes the catecholaminergic cellular demise. Such extra-neuronally consumed DOPA/dopamine may provide a theoretical model in agreement with the observations of transient overactivation of tyrosine hydroxylase (TH)-positive cells in the early or developing stage of age-correlated diseases preceding a dopamine defect, the mechanism of which remains unraveled. GSH-depleting electrophile(s) may be produced solely at the expense of DOPA and dopamine.

The abnormality in the determination of cell fate between cell death and growth is a predominant feature common to age-correlated diseases. The importance of the balance between proteases 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 over these decades. Paradoxical double roles for intracellular GSH, presumably depending on the quantity, in determining cell fate have been well documented with respect to both apoptotic and proliferative pathways. These include the implication of GSH levels in the regulation of thiol proteases caspase 3 and HIV-1 activities [11 12 25 26] in the case of HIV, allowing the assumption that sulfhydryl reactive-electrophiles may modulate progression towards death or survival via a mechanism involving the competition between GSH and thiol proteases, such as caspase 3, as a target of attack by electrophiles.

The hypothesis of GSH-depleting electrophiles may be advanced such that the production of electrophiles should depend on a certain biochemical mechanism involving ROS, NO, DOPA and catecholamines, considering the implication of the distorted regulation of cell fate in age-correlated diseases for the following reason. 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 of postmitotic neurons [27]. Caspase 3 inhibition alone can promote cell proliferation by preventing 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 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, NO, ROS, DOPA and catecholamines, to the regulation of cell fate. The possibility may be raised by accumulating evidence, as shown below, that a certain biochemical mechanism involving NO, ROS, DOPA and catecholamines operates a switch leading to the proliferation of non-neuronal cells in close proximity to degenerating catecholamine-producing cells. To go a step further, views may converge to one assumption that a distinct threshold for triggering a switch to cell death or growth depends on the absolute quantity of peroxynitrite (ONOO-) and DOPA/dopamine in a compartment.

The important experiments in cancer growth have revealed the capacity of ONOO- provided by host cells to quantitatively determine tumor growth and tumor-associated angiogenesis by inducing ONOO- provision of iNOS to cancer cells [47].

In addition, NGF-induced NOS not only reverses the apoptotic effect of 6-hydroxydopamine (6-OHDA) on catecholamine-producing cells but also exerts a potent angiogenic and proliferative action 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 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 in not only MPTP-treated animals [51] but also aged mammals [52]. These observations have led 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 leading to apoptosis, but if modified during the reaction with ONOO-, reverse apoptosis by inhibiting caspase 3 and elicit a promotion of mesodermal/mesenchymal cell proliferation. Such further modifications by ONOO-, though exemplified under pharmacological conditions in an *in vitro* study employing ROS-generating chemicals, may not occur in neuronal cells *in vivo*.

The implications of DOPA or dopamine quinones and related metabolites as endogenous cytotoxic electrophiles [53] derived from the reaction of DOPA or catecholamines with ONOO- [54] in age-correlated diseases have also been discussed as relevant to the cytoprotective role 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 attacked carbon, have been seen in diseased regions of age-correlated diseases and suggested to exert a cytoprotective action at least in part by catalyzing the conjugation of GSH to DOPA or dopamine quinones [33 57].

However, the physiochemical nature of DOPA- and dopamine-derived quinones may limit appreciation of the pathogenic roles of these quinones in age-correlated diseases displaying colocalization of DNA/RNA damage, protein modifications, functional deterioration of enzymes and concomitant alteration of GSH status. The capability of compounds to deplete GSH depends on their appropriateness as 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 rather by increasing electrophilicity on the carbon attacked or preserving active carbonyl than by ROS-generating redox cycling [58-63]. In this regard, none of topa, *p*-quinone of topa 6-OHDA-, *p*-quinone of 6-OHDA, dopaquinone, or dopamine *o*-quinone [53] can be considered strong causal candidates of the 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 reactivity of *p*-quinones of topa and 6-OHDA has been confirmed [53].

In general, for dopaquinones and dopamine quinones, GSH conjugation is considered neither to reduce the quinone capability of redox cycling nor to maintain the electrophilicity of the carbon on the 6-membered ring because of both a relatively stable benzene ring formed in conjugation and the chemical characteristics of GSH, with high nucleophilicity overwhelming a poor electron-donating property. Support for this view is also derived 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 detoxication of quinones depends on electrophile-responsive induction of DT-diaphorase,

glucuronosyl transferases, and sulfotransferases rather than GST-mediated elimination into extra-cellular spaces [63]. Specific up-regulation of GSTs may reflect an adaptive response to increased electrophiles that resist the activity of the export pump that would preferentially eliminate the GSH-conjugate to GSH itself, maintain electrophilicity beyond GSH conjugation and/or distort the cellular detoxication machinery.

Quinones serve as substrates for flavoenzymes, including NADPH-cytochrome P450-reductase, DT-diaphorase, NADPH-cytochrome b_5 reductase and NADH ubiquinone oxidoreductases, stimulating NAD(P)H oxidation [66]. Eventually, judging from all the features, quinones seem unlikely to cause either a severe decline in NAD^+-linked oxidation during aging to a greater extent than in Complex I or profound GSH depletion.

Thus, the search for a biologically active electrophile that steadily increases with age; that is produced at the expense of DOPA/dopamine; that depends on the quantity of ONOO- during both synthesis and further modification; that depletes GSH non-oxidatively as a good substrate for GSTs, such as α,β-unsaturated carbonyl compounds; that preserves active carbonyl group(s) or maintains the electrophilicity of the carbon undergoing conjugation to GSH; that displays reactivity towards both proteins and DNA/RNA; and that causes functional deterioration of $NAD(P)^+$-dependent oxidoreductases should attract much interest in the pursuit of understanding the common molecular basis underlying age-correlated diseases.

Indole-2,3-dione is a biologically active electrophile that is produced in every fundamental cycle (Fig. 1) and has the ability to interfere with electron-borne information via a complex mechanism, most notably by inhibiting the actions of ATP or NAD(P)H towards ANP receptors [67], peripheral benzodiazepine receptors (PBR) [68], or $NAD(P)^+$ -utilizing oxidoreductases [69], as mentioned below. It also inhibits the majority of detoxifying enzymes, including MAO[70], XO, DT-diaphorase (unpublished data), acetylcholine esterase [71] and phosphatases [72], suggesting that it affects not only detoxication but also the metabolism of purines, pterins and neurotransmitters. Of particular interest is that PBR and MAO B, both of which are targets of indole-2,3-dione inhibitory actions, localize to outer/inner mitochondrial membranes.

Among the various plants and bacteria, indole-2,3-dione has long been identified to be involved in metabolic pathways with distinctive enzymes, which are lost in animals. Having 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 displaying similarity to adenine may allow competition or replacement with ATP and NAD(P)H of this molecule, which together with the short wavelength light absorption, could result in disruption of the synchronization of these enzyme functions with the environment in response to 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 that 2 ONOO- are required for 1 indole-2,3-dione formation, suggesting the existence of a distinct threshold for the absolute quantity of ONOO- with respect to indole-2,3-dione formation in a given compartment. A rapid increase in indole-2,3-dione formation up to 10-fold [73] also supports the involvement of a free radical-mediated synthesizing process, although the participation of L-aromatic amino acid decarboxylase and/or MAO catalytic activity cannot be ruled out considering its chemical structure.

This sequential reaction process appears to be quite similar to that of lucigenin-based chemiluminescence [74], where 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. Photoemission arising from the lucigenin-based chemiluminescence system is basically distinguishable from that arising from other systems, including the luciferin derivative-based system, in that the former is driven by the energy drop during the oxidative decay of lucigenin and therefore does not directly quantify superoxide anions or singlet oxygen, although it appears to be roughly correlated with the rate of ROS generation within a certain window. In addition, the photoemission roughly reflecting the decaying 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 to be applied (Fig. 4). A drastic increase in photoemission that is consistently followed by a rapid fall to below basal levels or almost the nadir indicates that a rapid degradation of a 'parent' lucigenin due to s ROS supply exceeding a threshold terminates the sequential reaction. A similar phenomenon has been observed for indole-2,3-dione formation. The result shown in Figure 5 suggests that as long as the ROS generation rate never exceed 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 ROS-mediated degradation of lucigenin, but never precisely indicates ROS 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

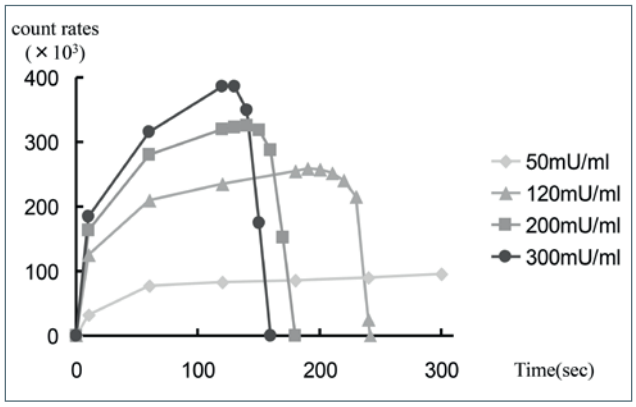

Figure 4. Lucigenin-based chemiluminescence in the hypoxanthine-XO ROS-generating system. Lucigenin (0.250 mM) was employed as the electron acceptor. The reaction was started by simultaneous addition of hypoxanthine and lucigenin. Photoemission was measured every seconds for 5 minutes in a luminometer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

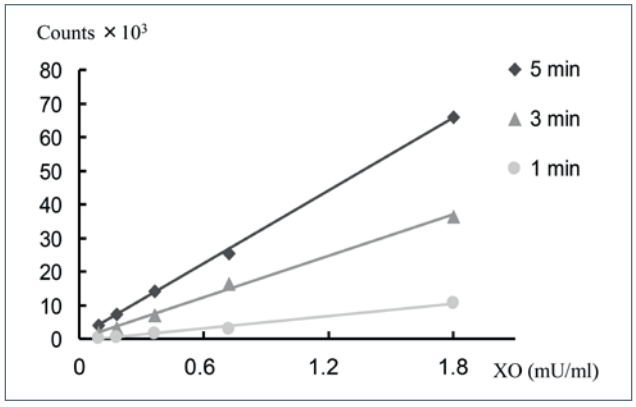

Figure 5. Lucigenin-dependent luminescence in the XO-hypoxanthine system at an XO concentrations as low as 0.1-2 mU. Each solid line indicates the counts integrated over 1, 3, and 5 minutes in a linear function of the XO concentration over a range from 0.1-2 mU of XO, corresponding to 10^{-10} –$2x10^{-9}$ M superoxide anion generation.

(Fig. 3), but rather it promotes DOPA/dopamine oxidative consumption, which would result in a dopamine defect if such reactions occur in extra-neuronal spaces and non-neuronal cells. Presuming that physiologically supplied Phe in not a limiting factor, the formation rate of indole-2,3-dione displays a steady state or a steady increase under normal physiological conditions, with a rapid fall that is only accomplished by the depletion of either DOPA or NO [69 73 75]. According to the similarity to the 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, it has a distinctive distribution into mammalian blood, tissues and organs at concentrations comparable to those of catecholamines, with higher concentrations being found 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 mM), seminal vesicle (~30 mM), kidney (~1.5 mM), lung (~0.8 mM), hippocampus (~0.9 mM), striatum (~0.8 mM) and cerebellum (~0.9 mM) [68 76], where nNOS is strongly expressed and colocalizes with TH-positive cells. Considering the selective vulnerability of the hippocampus or striatum in age-correlated apoptosis and the selective survival of the vas deferens and seminal vesicles in 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 during aging. This idea is compatible with the assumption induced by the previous observation that a several fold increase in indole-2,3-dione was elicited by specific agonists of 5-HT $_{2A/2C}$ receptors [75], which have long been known to mediate the potentiation of NOS and TH activities and have been recently appreciated as contributors to the ontogenetic determination of cell fate [77 78].

The unique physiochemical nature of indole-2,3-dione supports the view of this molecule as a strong candidate for depleting GSH while simultaneously causing mtDNA damage and protein modifications, as well as triggering a switch to cell death or growth. It is a heterocyclic quinonoid that harbors a benzene ring as a soft electrophile and 5-membered ring with active carbonyl groups as a hard electrophile, displaying a preference for proteins and DNA/RNA, respectively.

Local indole-2,3-dione concentration is expected to result from 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 of GSH and indole-2,3-dione in human blood (data not shown). As a soft electrophile, it undergoes GST-catalyzed conjugation with GSH at the 4 or 6 position under conditions of an ample GSH supply because of its appropriateness as a good substrate for GSTs. The resonance canonical form can also participate, suggesting that the electrophilicity of the carbon at 3 position may not be high enough to attack DNA/RNA because

of the relatively electro-withdrawing property of the glutathionyl group for the nucleophilic compound. These GSH conjugates are eliminated into extracellular fluids preferentially to GSH itself or otherwise, particularly under conditions of increased ONOO- production, during which the 6-glutathionyl conjugate can undergo nucleophilic displacement with the nitro group by ONOO- attack and subsequently nucleophilic attack by proteins, but not by DNA/RNA because of a comparative softness of electrophilicity (Fig. 9).

However, in the case that ONOO- attack of indole-2,3-dione precedes other modifications, the consequences become quite different and more detrimental (Fig. 9). Initial attack by ONOO- is expected to occur at the 5 position, but not at 4 or 6, to form 5-nitro substituted indole-2,3-dione displaying significantly enhanced electrophilicity on the carbon at 3, which 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) kept in contact with DNA/RNA, whereas the latter case could eventually lead to the generation of sulfonamides of indole-2,3-dione, the potent caspase 3 and 7 inhibitor [28], although the detailed reaction process awaits further examination. CO liberation from the indole-2,3-dione derivative covalently bound to DNA/RNA leads to functional deterioration of cytochromes because indole-2,3-dione has long been known to target MAO and PBR on the inner/outer mitochondrial membranes. Because the capability of the heterocyclic quinonoid indole-2,3-dione to undergo redox cycling is much less than that of catechol quinones with a 6-membered ring 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 could raise the possibility of the initial attack on this quinonoid by ONOO- leading to the generation of CO or caspase inhibitor as well as accessibility to nucleophilic macromolecule conjugates without a loss of electrophilicity on the carbon.

Support for the implication of indole-2,3-dione and its derivatives as contributing factors in age-correlated diseases has also been obtained from both their biological actions and observations of the specific increase in indole-2,3-dione up to 8-10-fold during stress [73] as well as in cancer [79], vasculopathy-based diseasses [80] and neurodegenerative diseases, with the levels correlated with the severity [81]. The close relationship between indole-2,3-dione and the stress response is supported by the distinct seasonal variations in rats showing the inverse correlation of its production with the open air temperature. The most potent physiological actions include inhibition of ANP receptor binding, its G-protein-mediated signaling, NAD(P)H-oxidizing flavoenzymes, peripheral benzodiazepine receptor (PBR) binding, and glucose tranport [82], allowing the assumption that it may modulate local inflammation, vasomotor tone, extracellular fluid volume and content, local steroidogenesis and metabolism of cholesterol and glucose, both directly and indirectly by discouraging benign actions of PBR and ANP regarding suppression of activation/induction of TH and iNOS. As mentioned above, the 5-nitro derivative has proved to be a moderate caspase 3 inhibitor with some antigenic properties, whereas further nucleophilic displacement with sulfhydryls at 5 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 the detoxifying enzymes, including MAO B, esterases, XO, phosphatases, glutathione S transferases, glutathione peroxidase, DT-diaphorase, and glucuronyl transferases (unpublished data). 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 it may resist enzymatic detoxication and also confer increased resistance of electrophiles, including catechol quinones, to the cellular detoxication machinery.

It is worthwhile to hypothesize that (1) the age-correlated increase in indole-2,3-dione, the endogenous inhibitor of both ANP binding/signaling and PBR binding/signaling, followed by an adaptive increase in plasma ANP, represents a molecular mechanism of aging. (2) 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 when the Phe supply is presumably not a limiting factor, (b) the rate of 5-nitro substitution for indole-2,3-dione is determined by the absolute quantity of both indole-2,3-dione and ONOO- in a given compartment as long as local GSH levels never exceed a putative threshold., then whether the age-correlated increase in indole-2,3-dione represents a proper aging mechanism that meets the above-mentioned demands of theory can be tested.

RESULTS

Age-related increase in indole-2,3-dione in rats

Indole-2,3-dione in plasma and urine display distinct diurnal and seasonal variations in humans and rats (unpublished data). Total indole-2,3-dione excretion in 24-hr urine within a given range of open air temperatures has been considered a stable indicator of the intrinsic production of indole-2,3-dione. Here we employed the averaged measurements in either July or January representing summer and winter, respectively.

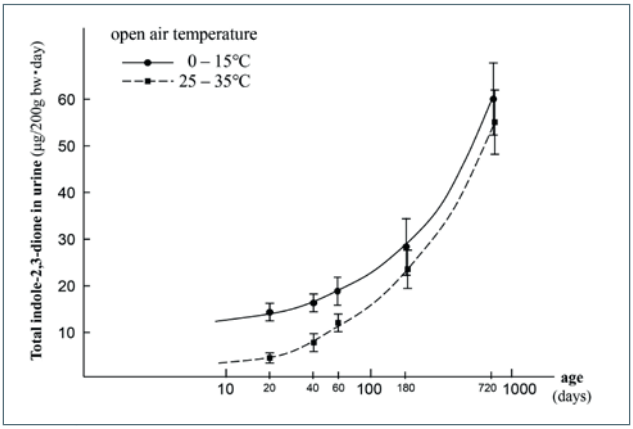

Figure 6. Age-associated increase in indole-2,3-dione, shown with seasonal variation. The dotted line indicates the measurements in winter in Japan in contrast with the solid line in summer.

The result shown in Fig. 6 indicates a positive association of indole-2,3-dione production with age, along with the influence of temperature on indole-2,3-dione production.

Relationship between GSH, GST activity in the liver and indole-2,3-dione excretion in 6-hr urine during fasting

It is well known the elicitation of GSH depletion to the highest extent in the liver is exerted by fasting, at least in part via depletion of the amino acids pool in the liver, which enhances the carcinogenic and cytotoxic effects of electrophiles. The results presented in Fig. 7 suggest a reciprocal relationship between hepatic GSH, GST activity and indole-2,3-dione excretion. Up to a 10-fold increase in indole-2,3-dione in 24-hr urine during fasting, which far exceeds the approximately

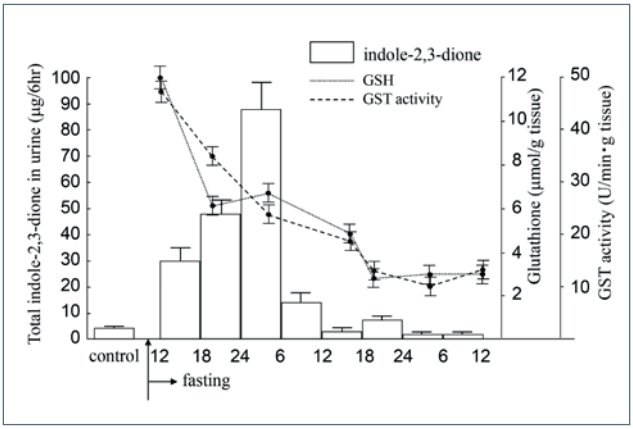

Figure 7. Relationship between the GSH and GST activities in liver and indole-2,3-dione excretion in 6-hr urine during fasting. The arrow indicates the time point at the beginning of fasting.

2-3-fold increase elicited by cold stress, has raised the question of whether the extent of increasing indole-2,3-dione during fasting can be accounted for by the burst of oxidative metabolism induced by stress. Although the author could not discern the cause of the increasing indole-2,3-dione solely from the result obtained herein, it is conceivable that increased indole-2,3-dione levels may be both a cause and a consequence of the systemic depletion of GSH caused by the depleted GSH pool during fasting. This view may be in agreement with the observation of the inverse correlation of indole-2,3-dione in human blood with liver GSH (data not shown).

Xanthine oxidase (XO) inhibition by indole-2,3-dione in the cell free system

To further confirm the interference with NAD(P)$^+$-utilizing flavoenzymes by indole-2,3-dione shown in the previous report, the flavoenzyme xanthine oxidase was employed, which is part of a large family of enzymes found in all phyla containing flavins and/or pterins as cofactors, and a component of circadian blue light input pathways and itself under circadian clock control, although the littermate xanthine dehydrogenase (XDH) does not exhibit circadian rhythmicity [83]. It catalyzes the conversion of hypoxanthine via xanthine to uric acid, which is involved in purine and pterin metabolism. XO is also capable of attracting electrons from NADH, although it does not have a preference for NAD$^+$ as a co-factor. As shown in Fig. 8, indole-2,3-dione is effective at concentrations as low as 20 mM for the suppression of ROS arising from the hypoxanthine-XO system. Judging from the extremely high concentrations in close proximity to catecholamine-producing cells, indole-2,3-dione may interfere with XO activity in the microcirculation, where XO-containing endothelial cells are in close contact with sympathetic nerve terminals.

DISCUSSION

Considering the unique physiological nature and distinct biological actions of indole-2,3-dione and its considered derivatives, the data presented herein seem to be in agreement with the demands of theory for a model of an aging mechanism based on a close link between detoxication, respiration, biosynthesis and the stress response, with particular primacy given to the impaired detoxication of electrophiles.

Cancer, vasculopathy-based diseases and neurodegenerative diseases have also been named age-correlated disease states due to their increasing incidence with age. They must share a molecular mechanism in line with a single mechanism of aging.

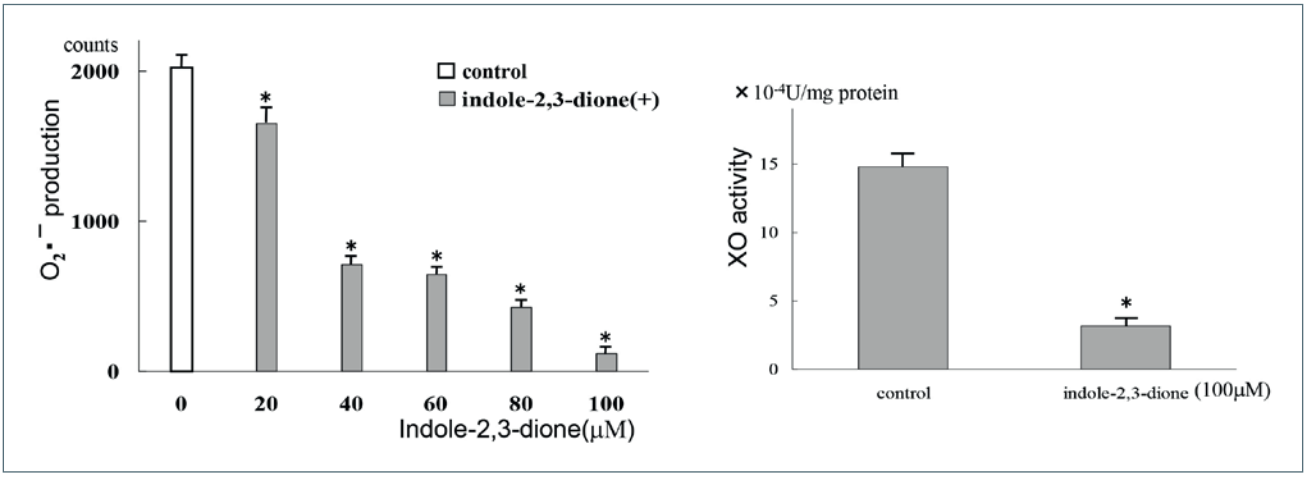

Figure 8. (left) Effect of indole-2,3-dione on hypoxanthine/XO $O_2 \cdot^-$ generation. MCLA was adopted as the electron acceptor because it has been established that MCLA-dependent luminescence in the XO-hypoxanthine system is a linear function of the XO concentration. The bar graphs show $O_2 \cdot^-$ production (n=5) in the presence or absence of indole-2,3-dione at each dose. Photon emission was measured every second in a luminometer and integrated for 5 minutes. An indole-2,3-dione solution was added to a reaction mixture in a cuvette at the start of incubation at 37° C for 5 minutes. Key: (*) $p < 0.01$ compared with the values obtained for the control without indole-2,3-dione. (Student's paired *t* test). (right) Effect of indole-2,3-dione on XO activity. An XO solution, hypoxanthine solution and 50 mM sodium phosphate buffer were mixed and incubated in the presence or absence of indole-2,3-dione (final concentration, 100 mM) at 37° C. The absorbance at 290 nm was measured between 5 and 20 minutes (n = 4). XO activity was calculated using the color absorbance coefficient: $1.22 \times 10^4 cm^{-1}$. Key: (*) $p < 0.005$ compared with the values obtained for the control without indole-2,3-dione. (Student's *t*-test).

In view of a selective vulnerability of catecholaminergic cells associated with induced expression of stress proteins and markers of inflammation in cells of mesodermal/mesenchymal origin within catecholaminergic terminal regions, a number of studies have centered on the pathological relevance of DOPA, catecholamines, ROS and RNS to age-correlated diseases. The important questions that remain include a profound dopamine defect preceding and exceeding catecholamine-producing cell demise and its association with the functional disruption of mitochondrial complex I throughout body.

Apart from the diet-derived portion, dopamine exclusively originates in DOPA produced by catecholamine-producing cells throughout life, with the exception of the early embryo depending on the maternal supply of DOPA, approximately up to E8.5 when the primordial TH positive cells appear. However, dopamine levels far exceeding those of norepinephrine depend on the supply from non-neuronal cells, 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 a steady increase in DOPA/dopamine oxidative consumption that also occurs in extraneuronal spaces and non-neuronal cells with age, rather than nitration-mediated deterioration of TH activity. Based on the assumption that diffusible molecules formed only at the expense of local DOPA/dopamine in the presence of ONOO- operate a switch leading to cell death and growth, the physiochemical nature of the electrophilic center, a possible modification resulting from the increased ONOO- and subsequent nucleophilic displacement and influence on detoxifying enzyme activity for a candidate electrophile indole-2,3-dione, are highlighted with particular emphasis on the relationship to GSH status and mtDNA damage in this study. The strict DOPA/dopamine requirement for indole-2,3-dione formation has been previously confirmed [69 73 75].

Support for the first working hypothesis has also been obtained 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, evidence that chemically divergent compounds induce the ROS- and caspase 3 activation-mediated common apoptotic pathway culminating in selective cell death of catecholamine-producing cells [85] indicates the involvement of the oxidation of DOPA or catecholamines in a common molecular mechanism underlying a selective vulnerability of catecholamine-producing cells. The observation that the inhibition of tyrosine hydroxylase alone suppresses dieldrin-induced ROS generation and DNA fragmentation [86] further confirms

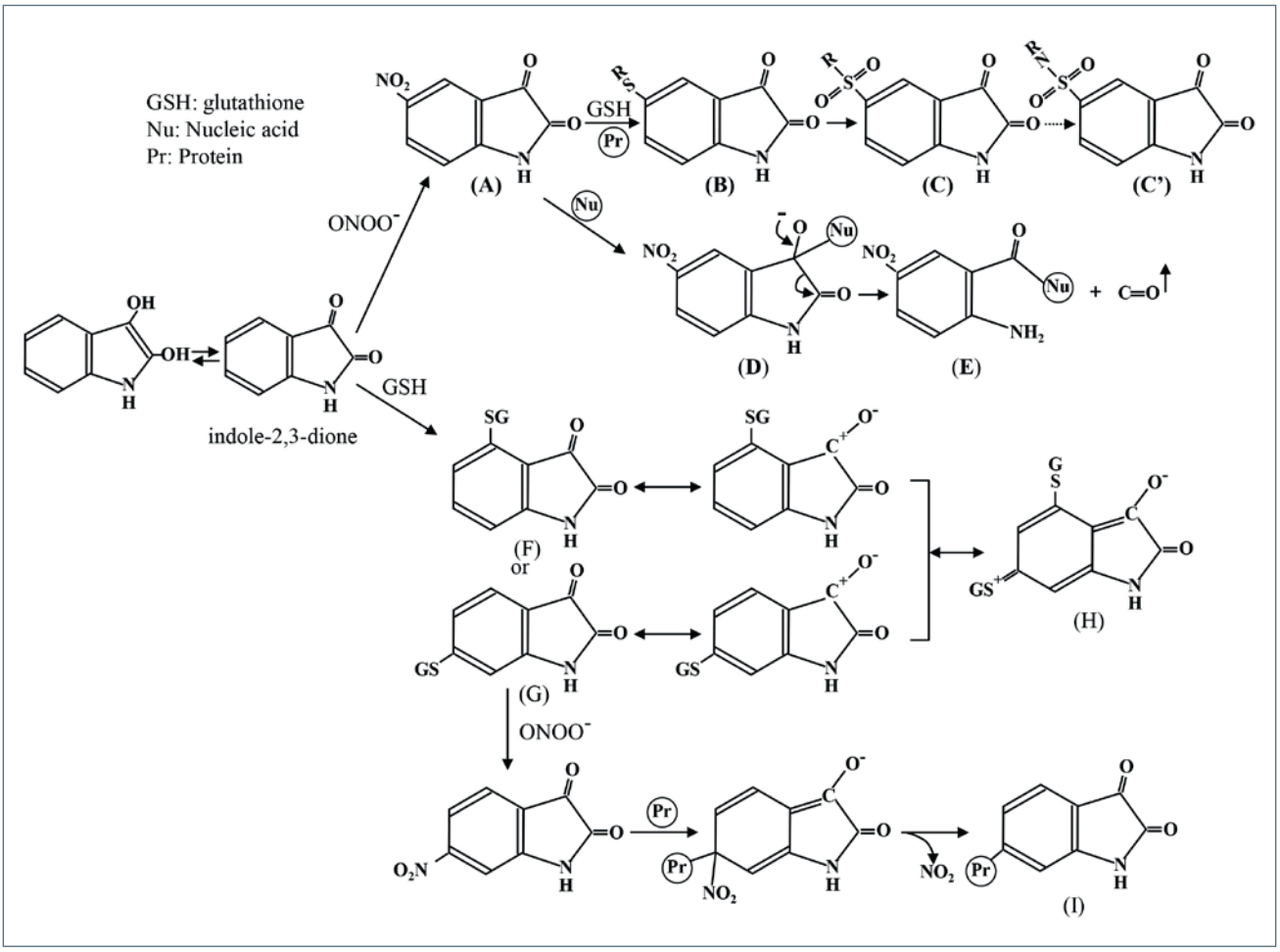

Figure 9. Schematic drawing of indole-2,3-dione and its derivatives shown with redox cycling.

the participation of DOPA or catecholamines in the apoptotic pathway and suggests an augmentation of ROS caused by oxidation of DOPA or catecholamines. Selective catecholaminergic cell death following microglial proliferation associated with the increase in HSP70 expression and inflammatory markers, such as induction of iNOS, which occurs within catecholaminergic terminal regions in METH-treated animals [87], suggests that diffusible small molecule(s) formed extra-neuronally at the expense of DOPA/catecholamines during 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 of 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 is modified in non-neuronal cells leading to the generation of a potent anti-apoptotic electrophile. In regard to both the formation of the above-mentioned electrophile at the expense of DOPA/dopamine and the subsequent modification, distinct thresholds for the absolute quantity of ONOO- in a given compartment should be expected. The former threshold originates from the requirement of 2 ONOO- for 1 electrophile, as mentioned previously, and the latter originates from the presumable competition with nucleophilic attack by cellular sulfhydryls. The existence of distinct thresholds for ONOO- common to apoptotic and proliferative pathways enables a synchronized operation of a switch to neuronal cell death and to non-neuronal cell proliferation, as well as age-correlated manifestations of specific pathological conditions, such as cancer, vasculopathy and neurodegeneration, which may be discriminated not qualitatively but quantitatively alone from normal aging and embryonic development. The sequence of events depends on NO and DOPA/dopamine alone.

In this regard, the great similarity of the indole-2,3-dione-generating process to lucigenin-based chemiluminescence

as well as the strict requirement for ONOO-mediated 5-nitro substitution on indole-2,3-dione for either the generation of a potent caspase 3 inihibitor [28] or CO liberation maintained in contact with DNA/RNA may position this electrophile as a strong candidate for triggering a switch to neuronal cell death associated with non-neuronal cell proliferation. Because conversion of the sulfide of indole-2,3-dione via sulfone to sulfonamide, the caspase 3 inhibitor, may require further ONOO- action following flavin-containing monooxygenase activity, the degree of ONOO- requirement for the generation of caspase 3 inhibitor seems much greater than that for the formation of the covalent bond to DNA/RNA. The observation that the synthesis of the potent caspase 3 inhibitor would not occur without 5-nitro substitution by ONOO- could account for the dependency of carcinogenesis and other pathological angiogenesis 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.

The finding that ONOO- attack following GSH conjugation at the 6 could result in protein modifications, but neither DNA/RNA damage nor caspase inhibition, may suggest that the intrinsic GSH level is critically implicated in the determination of cell fate, particularly in conditions of increased ONOO-.

The third working hypothesis is that the controversial problems in mitochondrial damage in aging would not be ascribable to oxidative stress by known oxygen toxic species, including 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 adopting GSH-depleting electrophiles, it might be accounted for by the potential lag time originating from the difference in probabilities and/or in accessibilities for the GSH-depleting electrophile to interfere with the binding site of the acceptor substrate (quinone) and to cause mtDNA damage. All aspects of the unique physiochemical property of the endogenous quinoid indole-2,3-dione seem to support the view of this electrophile as a strong candidate. Indole-2,3-dione itself may cause a decline in NAD^+-linked oxidation because it seems to withdraw electrons from NAD(P)H at the binding sites of $NAD(P)^+$ on oxidases. Increasing ONOO- production with age would result in nucleophilic substitution of the nitro group at the 6 position following GSH conjugation, leading to enzyme 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 predispose mitochondria to oxidative stress. Although what promotes the process leading to the generation of sulfonamides of indole-2,3-dione has not been verified, potent caspase 3 inhibitors, both flavin-containing monooxygenase activity [1] and a strong oxidizing compound are minimally required.

The interference with blue light reception by indole-2,3-dione may rise in importance, particularly in relation to the variability in 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 displaying some coincidence of the absorbance maximum with itself. In general, FAD-bound flavoenzymes display an absorbance maximum within the range from 380~450 nm, which is lost in the withdrawing electrons from substrate NAD(P)H. The circadian-rhythmic flavoenzyme XO capable of withdrawing electrons from NAD(P)H participates in blue light input pathways[83], which is blocked by allopurinol, an inhibitor for both XO and XDH lacking 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 cell free systems 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 products derived from the intrinsic mechanism of aging. The stress response initially arising from oxygen consumption in ectodermal cell terminates in the neutralization of electron flow by sulfur-containing compounds produced mainly in endodermal cells, through the detoxication (elimination) of electrophiles by detoxifying enzymes produced originally in mesodermal/mesenchymal cells. The deterioration with age of the integrating role for ANP and glucocorticoid derived mainly from the mesodermal cells in the regulation of a dynamic equilibrium between electrophiles and nucleophilic sulfhydryls, causes altered stress response outcomes in association with the disruption of electron-mediated transmission. This phenomenon may be exemplified by the case of indole-2,3-dione as mentioned below.

Considering the elicitation of the inhibitory action on G-protein-mediated signaling of ANP by indole-2,3-dione via interference with ATP binding sites [67], representing most conserved region throughout ANPs, the entire ANP family is presumably subject to indole-2,3-dione action. The extent of receptor binding inhibition may not differ significantly in a varied type of receptor

because of highly conserved extracellular portions [89]. Competitive inhibition by indole-2,3-dione of ANP binding should attract much interest in regard to the age-correlated manifestations originating from the disrupted ANP actions associated with a paradoxical elevation of plasma ANP levels. Because plasma ANP levels are determined simultaneously with the degradation by ecto-enzymes, the degree of clearance receptor binding and subsequent internalization rather than the achievement of G-protein-mediated signaling [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 due to disrupted ANP actions to suppress TH [91], iNOS [92], and vascular endothelial growth factor (VEGF) [93 94], concertedly with other biological actions of indole-2,3-dione derivatives. The similarity to vitamin K [95] in chemical structure may confer increasing importance to this electrophile in pathologies associated with aging.

Indoel-2,3-dione is an inhibitor of the majority of the detoxifying enzymes, including XO, MAO, esterases, GSTs and DT-diaphorase (unpublished data), which 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 accounted for, in part, by interfering electrophiles, such as indole-2,3-dione. Some controversial observations of XO activation in relation to vasculopathy may derive in part from interference by electrophile(s). GST inhibition combined with GSH depletion by indole-2,3-dione may cause apoptotic neuronal death. In contrast, under conditions of robust ONOO- production in cells of mesodermal/mesenchymal origin, 5-nitro substitution makes indole-2,3-dione resistant to benign GSH conjugation at 4 or 6 positions, leading to a gradual recovery of GSH.

Interference with cytochromes and flavoenzymes by indole-2,3-dione could result in deterioration of transport and metabolism of cholesterol and glucose, which together with inhibition of PBR binding and glucose transport, may allow the manifestation of impaired steroidogenesis and insulin resistance during senescence.

After birth, NOS and TH are both considered the link between neural activity and activity-dependent regional vasomotor regulation. However, during early embryonic development, the timing and the sites of a 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 during organogenesis associated with robust vascularization. At the same developmental stage, oxygen tension dramatically increases, reflecting the rapid establishment of the O_2-supplying cardiovascular system. The view 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, especially 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 otherwise lethality. Although the biological actions of multifunctional peptides, ANPs, encompass the regulation of extra-cellular fluid volume and content and acute and chronic vasomotor tone, the timing and the 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, particularly by modulating a certain signal from iNOS-expressing cells [98]. In addition, a close but mutually exclusive relationship of ANP to TH has also been supported by anatomical evidence during embryonic development [99-105], particularly in regard to the establishment of the mature heart rate and conducting system, genetic targeting studies centered on hypoxia-inducible transcription factors [106] and the localization of ANP and its receptors in the nervous system of adults [107]. The ANP gene harboring both AP1 and CRE within its promoter region [108], very similarly to the TH gene, may allow the assumption that ANP responds to GSH-depleting electrophiles and, synergistically with TH, modulates cell fate or cycle and maintains a dynamic equilibrium for both local oxygen tension and oxygen-containing electrophiles. The regulatory role of ANP in blood pressure, peripheral resistance or the sodium/water balance may be merely a secondary product derived from its above-mentioned intrinsic role.

PBR constitutes one of the two cholesterol transport mechanisms as a mediator of the acute stimulation of steroidogenesis by hormones. Predominantly localized on outer/inner mitochondrial membrane contact sites in steroidogenic cells of gonads, adrenals, placenta and brain, PBR functions as a mitochondrial cholesterol channel, modulates local and circulating glucocorticoid levels and in some cases promotes cell proliferation. PBR, which is 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 form iNOS during early embryogenesis. At the beginning of catecholamine production intrinsic to the embryo from E8.5 to E14, when peak expression is achieved, in association with the establishment of the cardiovascular system and ANP expression, the DOPA supply switches from maternal to embryonic primordial TH. Thereafter,

the produced indole-2,3-dione is achieved during each fundamental cycle, causing the deterioration of glucose transport, ANP action and glucocorticoid generation via an ANP-or PBR-dependent mechanism and CO-mediated cytochrome inhibition, which in turn concertedly disrupt the intrinsic mechanism to recover GSH depleted by indole-2,3-dione itself. The 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 stress-induced combustion of bi-radical oxygen is transformed to electrophilicity on adjacent atom(s), such as carbon, which in turn conveys, transforms or augments the information via AP-1-mediated induction of proteins and/or is completely neutralized or detoxified by the intrinsic sulfur displaying similarity for the electron configuration to oxygen. Such an energy flow in the form of electron-mediated information from ectodermal cells in the nervous system to endodermal cells in the liver cells may be manipulated by glucocorticoids produced in mesodermal/mesenchymal cells.

A proper transmission of electron-mediated information beyond cell membranes is indispensable for living. However, in the case electrophiles originating from oxygen consumption that 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 testified from as early as the first cleavage divisions of the embryo by the well-known 2 cell block phenomenon, suggesting that disrupted transmission of electron-mediated information caused by impaired detoxication of electrophiles may affect the determination of cell death and growth. The life span of organisms may be limited not by aging of individual cells but by the development of distortions in operating a switch between cell death and growth with age, which should originate in the impaired detoxication of electrophiles that endure time and distance much greater than free radicals, which bear short-lived information in the form of an unpaired electron in the outer orbitals.

A fundamental feature that is common to age-correlated diseases is a distorted determination of cell fate between death and proliferation, which should not occur as a consequence of 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 the neural activities of sympathetic and parasympathetic nerves after the establishment of the peripheral nervous system. However, during early development, iNOS contributes to cell proliferation and apoptotic death, presumably in cooperation with subsequently induced TH, by bearing the information derived from electrons, which may descend from lower organisms such as bacteria. According to the theory of the bacterial origin of mitochondria, mitochondria are considered to be quite sensitive to electron-borne information mediated by electron-donating molecules or compounds bearing unpaired electron(s), such as NO.

Animals, all of which are predators, have evolved light receptor oxidoreductases adapted for detoxication (elimination) [1] that are indispensable for dietary digestion and excretion, rather than photosynthesis and immobilization of inorganic compounds. Incomplete detoxification (elimination) of the electrophile indole-2,3-dione that originates from the essential amino acid Phe and in the production rate depends on NOS and TH activities, and it could result in a decline in the ability of blue light receptor oxidases to withdraw electrons from NAD(P)H and the efficiency of ADP/ATP-linked reactions, even in mitochondria of bacterial origin. It could also affect circadian clocks because light input pathways in animals exclusively depend on blue light reception. These phenomena may be the only reason why life is always limited.

METHODS

Animals

All procedures were approved by The Animal Research Committee and meet the Guideline for the Care and Use of Laboratory Animals of the School of Medicine, University of Tokyo. Male Wistar rats (Japan Biological Materials Center, Tokyo, Japan) were housed in metabolic cages, had easy access to tap water and food pellets with the exception of the fasting period and were maintained under a 12-hr light cycle throughout an acclimatization period of at least 5 days. Rat urine samples were collected daily at 14:00 for 24-hr urine or at 2:00, 8:00, 14::00 and 20:00 for 6-hr urine.

Reagents

XO (grade III buttermilk), modified Hank's balanced salt solution (mHBSS) (without phenol red, calcium or magnesium), hypoxanthine and lucigenin were all purchased from Sigma Chemical (St. Louis, MO). The 2-methyl-6-(*p*-methoxyphenyl)-3,7- dihydroimidazo[1,2-a] pyrazin-3-one (MCLA) was purchased from Tokyo Kasei (Tokyo, Japan).

Chemiluminescence assay using the hypoxanthine-xanthine oxidase (XO) system XO activity (final concentration 10 mU/mL) in the presence or absence of indole-2,3-dione was measured by a luminescence assay adopting either 0.5 m M MCLA as the electron

acceptor and 0.1 mM hypoxanthine as the substrate with mHBSS (pH 7.4) to a total volume of 2.0 mL. Each reaction mixture other than the substrate and MCLA was incubated at 37° C for 5 minutes. The reaction was started by the simultaneous addition of hypoxanthine and MCLA. Photon emission 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. MCLA-dependent luminescence in the XO-hypoxanthine system is a linear function of the XO concentration. For lucigenin-based chemiluminescence, 0.025 mM lucigenin was employed throughout 0.1-300 mU XO activity.

Spectrophotometric assay for XO activity

XO activities in the presence or absence of indole-2,3-dione (final concentration, 100 mM) were measured by continuous monitoring of the formation of uric acid at 290 nm. XO enzyme solution (final concentration, approximately 10 mU/mL), hypoxanthine (final concentration, 50 mM) and 50 mM sodium phosphate buffer (pH 7.8) were mixed in a cuvette (final volume, 3 mL). The absorbance at 290 nm was monitored between 5 and 20 minutes after incubation at 37° C.

Determination of total GSH content and GST activity in liver

Tissues were homogenized in ice-cold 0.25 M sucrose (w/v-1/10). GSH was assayed as a major non-protein sulfhydryl according to established procedures as described previously [6 109]. Hepatic cytosolic GST activity was measured spectrophotometrically with 1-choloro-2,4-dinitrobenzoic acid as the substrate according to previously methods described [110].

Extraction of indole-2,3,-dione

As described previously [69 73], rat urine (1 ml) was diluted in 5 ml of distilled water and acidified with 6 M HCl to pH 1. The urine sample was then heated for 10 min in a boiling water bath to solubilize the urine sediment. After cooling at room temperature, indole-2,3-dione was extracted with 10 ml of ethyl acetate. The organic layer was then evaporated under a stream of nitrogen, and the residue was dissolved in 0.3 ml of methanol and then diluted in 5 ml of 50 mM potassium phosphate buffer, pH 7.4. Indole-2,3-dione was extracted using a disposable solid-phase column (Mega Bond Elut C18 column, Varian, Harbor city, CA, USA), and the eluate was analyzed by reversed-phase HPLC.

HPLC analyses

Reversed-phase HPLC analyses were performed using a Hitachi 655A chromatograph (Hitachi, Tokyo, Japan) as described previously [69 73]. Briefly, partial purification of indole-2,3-dione was carried out using a Shodex ES-502C column (100 7.6 mm I.D., 9.0 µm particle size; Showa Denko, Tokyo, Japan) under the following conditions: mobile phase, 50 mM potassium phosphate buffer (pH 7.4)-acetonitrile (85:15, V/V); flow rate, 1.0 ml/min; 50° C. The final HPLC analysis was carried out using a Kaseisorb LC ODS Super column (250 4.6 mm I.D., 5 µm particle size and 120 Å pore size; Tokyo Chemical Industries, Tokyo, Japan).

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Conflict of interest

The Authors have no conflict of interest.

References

1 Jacoby WB, Ziegler DM. *The enzymes of detoxication.* J Biol Chem 1990;265:20715-8.

2 Edwards JL, King WA, Kawarsky SJ, et al. *Responsiveness of early embryos to environmental insults: potential protective roles of HSP70 and glutathione.* Theriogenology 2001;55:209-23.

3 Beck LV, Rieck VD, Duncan B. *Diurnal variation in mouse and rat liver sulfhydryl*. Proc Soc Exp Biol Med 1958;97:229-31.

4 Calcurtt G, Ting M. *Diurnal variations in rat tissue disulphide levels*. Naturwissenschaften 1969;56:419-20.

5 Farooqui MYH, Ahmed AE. *Circadian periodicity of tissue glutathione and relationship with lipid peroxidation in rats*. Life Sci 1984;34:2413-8.

6 Calcutt, G. *Diurnal variations in rat blood glutahione levels.* Naturwissenschaften 1967;54:120.

7 Harman D. *Aging: a theory based on free radical and radiation chemistry*. J Gerontol 1956;11:298-300.

8 Miquel J, Economos AC, Fleming JE, et al. *Mitochondrial role in cell aging*. Exp Gerontol 1980;15:575-91.

9 Genova ML, Castelluccio C, Fato R, et al. *Major changes*

in complex I activity in mitochondria from aged rats may not be detected by direct assay of NADH: coenzyme Q reductase. Biochem J 1995;311:105-9.

10 Moore GA, Orrenius S, O'brien PJ. *Menadione (2-methyl-1,4-naphthoquinone)-induced Ca^{2+} release from rat liver mitochondria is caused by NAD(P)H oxidation.* Xenobiotica 1986;16:873-82.

11 Herzenberg LA, De Rosa SC, Dubs JG, et al. *Glutathione deficiency is associated with impaired survival in HIV disease.* Proc Natl Acad Sci USA 1997;94:1967-72.

12 Aillet F, Masutani H, Elbim C, et al. *Human immunodeficiency virus induces a dual regulation of Bcl-2, resulting in persistent infection of CD4(+) T-or monocytic cell lines*. J Virol 1998;72:9698-705.

13 Dröge W, Schulze-Osthoff K, Mihm S, et al. *Functions of glutahione and glutathione disulfide in immunology and immunopathology*. FASEB J 1994;8;1131-8.

14 Raidel SM, Haase C, Jansen NR, et al. *Targeted myocardial transgenic expression of HIV Tat causes cardiomyopathy and mitochondrial damage*. Am J Physiol Heart Circ Physiol 2002;282:H1672-8.

15 Faustman-Watts EM, Namkung MJ, Juchau MR. *Modulation of the embryotoxicity in vitro of reactive metabolites of 2-acetylaminofluorene by reduced glutathione and ascorbate and via sulfation.* Toxicol Appl Pharmaol 1986;86:400-10.

16 Harris C, Namkung MJ, Juchau MR. *Regulation of glutathione in rat embryos and visceral yolk sacs and its effect on 2-nitrosofluorene-induced malformations in the whole embryo culture system.* Toxicol Appl Pharmacol 1987;88:141-52.

17 Stark KL, Harris C, Juchau MR. *Influence of electrophhilic character and glutathione depletion on chemical dysmorphogenesis in cultured rat embryos*. Biochem Pharmacol 1989;38:2685-92.

18 Harris C, Stark KL, Juchau MR. *Glutathione status and the incidence of neural tube defects elicited by direct acting teratogens in vitro*. Teratology 1988;37:577-90.

19 Slott VL, Hales BF. *Enhancement of the embryotoxicity of acrolein, but not phosphoramide mustard, by glutathione depletion in rat embryos in vitro*. Biochem Pharmacol 1987;36:2019-25.

20 Slott VL, Hales BF. *Protection of rat embryos in culture against the embryotoxicity of acrolein using exogenous glutathione.* Biochem Pharmacol 1987;36:2187-94.

21 Hockenbery DM, Oltvai ZN, Yin XM, et al. *Bcl-2 functions in an antioxidant pathway to prevent apoptosis*. Cell 1993;75:241-51.

22 Kuchel O, Shigetomi S. *Defective dopamine generation from dihydoxyphenylalanine in stable essential hypertensive patients*. Hypertension 1992;19:634-8.

23 Ara, J, Przedborski S, Naini AB, et al. *Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).* Proc Natl Acad Sci USA 1998;95:7659-63.

24 Basu S, Dasgupta PS. *Decreased dopamine receptor expression and its second messenger cAMP in malignant human colon tissue*. Dig Dis Sci 1999;44:916-21.

25 Hampton MB, Fadeel B, Orrenius S. *Redox regulation of the caspases during apoptosis.* Ann NY Acad Sci 1998;854:328-35.

26 Davis DA, Yusa K, Gillim LA, et al. *Thiotransferase (glutaredoxin) is detected within HIV-1 and can regulate the activity of glutathionylated HIV-1 protease in vitro*. J Biol Chem 1997;272:25935-40.

27 Blaschke AJ, Staley K, Chun J. *Widespread programmed cell death in proliferative and postmitotic regions of the fatal cerebral cortex.* Development 1996;122:1165-74.

28 Lee D, Long SA, Adams JL, et al. *Potent and selective nonpeptide inhibitors of caspase 3 and 7 inhibit apoptosis and maintain cell functionality*. J Biol Chem 2000;275:16007-14.

29 Jimenez B, Volpert OV, Crawford SE, et al. *Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1*. Nature Med 2000;6:41-8.

30 Schena M, Mulatero P, Schiavone D, et al. *Vasoactive hormones induce nitric oxide synthase mRNA expression and nitric oxide production in human endothelial cells and monocytes*. Am J Hypertens 1999;12:388-97.

31 Bonfoco E, Krainc D, Ankarcrona M, et al. *Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures.* Proc Natl Acad Sci USA 1995;92:7162-6.

32 Patel RP, Moellering D, Murphy-Ullrich J, et al. *Cell signaling by reactive nitrogen and oxygen species in atherosclerosis.* Free Radic Biol Med 2000;28:1780-94.

33 Weingarten P, Zhou Q-Y. *Protection of intracellular dopamine cytotoxicity by dopamine disposition and metabolism factors*. J Neurochem 2001;77:776-85.

34 Pappano AJ. *Ontogenetic development of autonomic neuroeffector transmission and transmitter reactivity in embryonic and fetal hearts.* Pharmacol Rev 1977;29:3-33.

35 Teitelman G, Baker H, Joh TH, et al. *Appearance of catecholamine-synthesizing enzymes during development of rat sympathetic nervous system: possible role of tissue environment*. Proc Natl Acad Sci USA 1979;76:509-13.

36 Teitelman G, Gershon M, Rothman TP, et al. *Proliferation and distribution of cells that transiently express a catecholaminergic phenotype during development in mice and rats*. Develop Biol 1981;86:348-55.

37 Hart B, Stanford GG, Ziegler MG, et al. *Catecholamines: study of interspecies variation*. Crit Care Med 1989;17:1203-22.

38 Baker H, Farbman AI. *Olfactory afferent regulation of the dopamine phenotype in the fetal rat olfactory system.* Neuroscience 1993;52:115-34.

39 Son JH, Min N, Joh TH. *Early ontogeny of catecholaminergic cell lineage in brain and peripheral neurons monitored by tyrosine hydroxylase-lac Z transgene*. Mol Brain Res 1996;36:300-8.

40 Kobayashi K, Morita S, Sawada H, et al. *Targeted disruption of the tyrosine hydroxylase locus results in severe catechoamine depletion and perinatal lethality in mice*. J Biol Chem 1995;270:27235-43.

[41] Huang MH, Firend DS, Sunday ME, et al. *An intrinsic adrenergic system in mammalian heart.* J Clin Invest 1996;98:1298-303.

[42] Ebert SN, Thompson RP. *Embryonic epinephrine synthesis in the rat heart before innervation*. Circ Res 2001;88:117-24.

[43] Breidbach O, Urbach R. *Embryonic and postembryonic development of serial homologous neurons in the subesophageal ganglion of Tenebrio molitor (insecta: coleoptera).* Microsc Res Tech 1996;35:180-200.

[44] Arnhold S, Anderssen C, Bloch W, et al. *NO synthase-II is transiently expressed in embryonic mouse olfactory receptor neurons.* Neuroscience Lett 1997;229:165-8.

[45] Heneka MT, Feinstein DL. *Expression and function of inducible nitric oxide synthase in neurons.* J Neuroimmunol 2001;114:8-18.

[46] Peunova N, Enikolopov G. *Nitric oxide triggers a switch to growth arrest during differentiation of neuronal cells.* Nature 1995;375:68-73.

[47] Konopka T, Barker JE, Bamford TL, et al. *Nitric oxide synthase II gene disruption: implication for tumor growth and vascular endothelial growth factor production*. Cancer Res 2001;61:3182-7.

[48] Calza L, Giardino L, Giuliani A, et al. *Nerve growth factor control of neuronal expression of angiogenetic and vasoactive factors*. Proc Natl Acad Sci USA 2001;98:4160-5.

[49] Shimoke K, Chiba, H. *Nerve growth factor prevents 1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine-induced cell death viaa the Akt pathway by suppressing caspase-3-like activity using PC12 cells: relevance to therapeutical application for Parkinson's disease*. J Neurosci Res 2001;63:402-9.

[50] Przedborski S, Jackson-Lewis V, Yokoyama R, et al. *Role of neuronal nitric oxide in 1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurotoxicity*. Proc Natl Acad Sci USA 1996;93:4565-71.

[51] Smith MA, Richey Harris PL, Sayre LM, et al. *Widespread peroxynitrite-mediated damage in Alzheimer's disease*. J Neurosci 1997;17:26537.

[52] Uttenthal LO, Alonso D, Fernández AP, et al. *Neuronal and inducible nitric oxide synthase and nitrotyrosine immunoreactivities in the cerebral cortex of the aged rat*. Microsc Res Tech 1998;43:75-88.

[53] Graham DG, Tiffany SM, Bell Jr. WR, et al. *Autoxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds towards C1300 neuroblastoma cells in vitro*. Mol Pharmacol 1978;14:644-53.

[54] Hastings TG, LaVoie MJ. *Peroxynitrite- and nitrite-induced oxidation of dopamine: implication for nitric oxide in dopaminergic cell loss*. J Neurochem 1999;73:2546-54.

[55] Bergelson S, Pinkus R, Daniel V. *Intracellular glutahione levels regulate Fos/Jun induction and activation of glutathione S-transferase gene expression*. Cancer Res 1994;54:36-40.

[56] Friling RS, Bergelson S, Daniel V. *Two adjacent AP-1-like binding sites form the electrophile-responsive element of the murine glutahione S-transferase Ya subunit gene*. Proc Natl Acad Sci USA 1992;89:668-72.

[57] Baez S, Aguilar SA, Widersten M, et al. *Glutathione transferases catalyse the detoxication of oxidized metabolites (o-quinones) of catecholamines and may serve as an antioxidant system preventing degenerative cellular processes.* Biochem J 1997;324:25-8.

[58] Boyland BE, Chasseaud L.F. *Enzymes catalyzing conjugations of glutathione with $\alpha\beta$-unsaturated carbonyl compounds.* Biochem J 1968;109:651-61.

[59] Boyland E, Chasseaud LF. *The effect of some carbonyl compounds on rat liver glutathione levels.* Biochem Pharmacol 1970;19:1526-8.

[60] Chuang AHL, Mukhtar H, Bresnick E. *Effects of diethyl maleate on aryl hydrocarbon hydroxylase and on 3-methylcholanthrene-induced skin tumorigenesis in rats and mice*. J Natl Cancer Inst 1978;60:321-5.

[61] Plummer JL, Smith BR, Sies H, et al. *Chemical depletion of glutathione in vivo.* Methods Enzymol 1981;77:50-9.

[62] Rocci L, Moore GA, Orrenius S, et al. *Quinone toxicity in hepatocytes without oxidative stress.* Archiev Biochem Biophys 1986;251:25-35.

[63] Öllinger K, Kägedal K. *Induction of apoptosis by redox-cycling quinones*. Subcellular Biochemistry 2002;36:151-70.

[64] Buffinton GD, Öllinger K, Brunmark A, et al. *DT-diaphorase-catalysed reduction of 1,4-naphthoquinone derivatives and glutahionyl-quinone conjugates*. Biochem J 1989;257:561-71.

[65] Shi MM, Kugelman A, Iwamoto T, et al. *Quinone-induced oxidative stress elevates glutathione and induces g-glutamylcysteine synthetase activity in rat lung epithelial L2 cells.* J Biol Chem 1994;269:26512-7.

[66] Chesis PL, Levin DE, Smith MT, et al. *Mutagenicity of quinones: pathways of metabolic activation and detoxification*. Proc Natl Acad Sci USA 1984;81:1696-700.

[67] Medvedev AE, Sandler M, Glover G*. Interaction o isatin with type-A natriuretic peptide receptor: possible mechanism.* Life Sci 1998;62:2391-8.

[68] Armando I, Levin G, Barontini M. *Stress increases endogenous benzodiazepine receptor ligand-monoamine oxidase inhibitory activity (tribulin) in rat tissues.* J Neural Transm 1988;71:29-37.

[69] Tozawa Y, Matsushima K. *Peripheral 5-HT_{2a}-receptor-mediated formation of an inhibitor of atrial natriuretic peptide binding involves inflammation.* Eur J Pharmacol 2002;440:37-44.

[70] Medvedev AE, Clow A, Sandler M, et al. *Isatin: a link between natriuretic peptides and monoamines?* Biochem Pharmacol 1996;52:385-91.

[71] Kumar R, Bansal RC, Mahmood A. *Isatin, an inhibitor of acetylcholinesterase activity in rat brain*. Biog Amines 1993;9:281-9.

[72] Singh B, Sharma R, Sareen KN, et al. *Isatin enzyme interactions*. Enzyme 1977;22:256-61.

[73] Tozawa Y, Ueki A, Manabe S, et al. *Stress-induced increase in urinary isatin excreton in rats: reversal by both dexamethasone and a-methyl-p-tyrosine*. Biochem Pharmacol 1998;56:1041-6.

[74] Liochev SI, Fridovich I. *Lucigenin as mediator of superoxide production: revised*. Free Radical Biol Med 1998;25:926-8.

[75] Tozawa Y, Ueki A, Shimosawa T, et al. *5-HT$_{2A/2C}$ receptor agonist-induced increase in urinary isatin excretion in rats: reversal by both diazepam and dexamethasone*. Biochem Pharmacol 1999;58:1329-34.

[76] Watkins P, Clow A, Glover V, et al. *Isatin, regional distribution in rat brain and tissues.* Neurochem Int 1990;17:321-3.

[77] Hamada S, Senzaki K, Hamaguchi-Hamada K, et al. *Localization of 5-HT2A receptor in rat cerebral cortex and olfactory system revealed by immunohistochemistry using two antibodies raised in rabbit and chicken.* Mol Brain Res 1998;54:199-211.

[78] Lauder JM, Wilkie MB, Wu C, et al. *Expression of 5-HT$_{2a}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors in the mouse embryo.* Int J Devl Neuroscience 2000;18:653-62.

[79] Maki, Y. *Study on urines of cancer patients*. Nihon Yakurigaku Zasshi (Japanese) 1959;55:1514-21.

[80] Hamaue N. *Endogenous monoamine oxidase (MAO) inhibitor (tribulin-like activity) in the brain and urine of stroke-prone SHR*. Biog Amines 1992;8:401-12.

[81] Hamaue N. *Pharmacological role of isatin, an endogenous MAO inhibitor*. Yakurigaku Zasshi (Japanese) 2000;120:352-62.

[82] Mousavi Gargari ML, Bansal RC, Singh K, et al. *Inhibition of glucose transport in human erythrocytes by 2,3-dioxoindole (isatin).* Experientia 1994;50:833-6.

[83] Deng TS, Roenneberg T. *The flavo-enzyme xanthine oxidase is under circadian control in the marine alga Gonyaulax.* Naturwissenschaften 2002;89:171-5.

[84] Lee MR. *Dopamine and the kidney: ten years on*. Clin Sci 1993;84:357-75.

[85] Chun HS, Gibson GE, DeGiorgio LA, et al. *Dopaminergic cell death induced by MPP$^+$, oxidant and specific neurotoxicants shares the common molecular mechanism*. J Neurochem 2001;76:1010-21.

[86] Kitazawa M, Anantharam V, Kanthasamy AG. *Dieldrin-induced oxidative stress and neurochemical changes contribute to apoptotic cell death in dopaminergic cells*. Free Radical Biol Med 2001;31:1473-85.

[87] Escubedo E, Guitart L, Sureda FX, et al. *Microgliosis and down-regulation of adenosine transporter induced by methamphetamine in rats*. Brain Res 1998;814:120-6.

[88] Lenaz G, Bovina C, Castelluccio C, et al. *Mitochondrial complex I defects in aging*. Mol Cell Biochem 1997;174:329-33.

[89] Takei Y, Hirose S. *The natriuretic peptide system in eels: a key endocrine system for euryhalinity?* Am J Phiol Regulatory Integrative Comp Physiol 2002;282:R940-51.

[90] Brenner BM, Ballermann BJ, Gunning ME, et al. *Diverse biological actions of atrial natriuretic peptide.* Physiol Rev 1990;70:665-99.

[91] Vatta MS, Rodríguez-Fermepín M, Durante G, et al. *Atrial natriuretic factor inhibits norepinephrine biosynthesis and turnover in the rat hypothalamus*. Regulatory Peptides 1999;85:101-7.

[92] Kiemer AK, Vollmar AM. *Autocrine regulation of inducible nitric-oxide synthase in macrophages by atrial natriuretic peptides*. J Biol Chem 1998;273:13444-51.

[93] Pedram A, Razandi M, Hu RM, et al. *Vasoactive peptides modulate vascular endothelial cell growth factor production and endothelial cell proliferation and invasion*. J Biol Chem 1997;272:17097-103.

[94] Pedram A, Razandi M, Levin ER. *Natriuretic peptides suppress vascular endothelial cell growth factor signaling to angiogenesis.* Endocrinology 2001;142:1578-86.

[95] Matsubara T, Touchi A, Hirauchi T, et al. *Depression of liver microsomal vitamin K epoxide reductase activity associated with antibiotic-induced coagulopathy.* Biochem Pharmacol 1989;38:2693-701.

[96] Yamada K, Noda Y, Komori Y, et al. *Reduction in the number of NADPH-diaphorase-positive cells in the cerebral cortex and striatum in aged rats.* Neuroscience Res 1996;24:393-402.

[97] Hales B, Huang C. *Regulation of the Y_q subunit of glutahione S-transferase p in rat embryos and yolk scs during organogenesis.* Biochem Pharmacol 1994;47:2029-37.

[98] Kiemer AK, Vollmar AM, Bilzer M, et al. *Atrial natriuretic peptide reduces expression of TNP-α mRNA during reperfusion of the rat liver upon decreased activation of NF-kB and AP-1.* J Hepatology 2000;33:236-46.

[99] Bloch KD, Seidman JG, Naftilan JD, et al. *Neonatal atria and ventricles secrete atrial natriuretic factor via tissue-specific sevretory pathways*. Cell 1986;47:695-702.

[100] Thompson RP, Simson JAV, Currie MG. *Atriopeptin distribution in the developing rat heart.* Anat Embryol 1986;175:227-33.

[101] Gardner DG, Deschepper CF, Ganong WF, et al. *Extra-atrial expression of the gene for atrial natriuetic factor*. Proc Natl Acad Sci USA 1986;83:6697-701.

[102] Wei Y, Rodi CP, Day ML, et al. *Developmental changes in the rat atriopeptin hormonal system.* J Clin Invest 1987;79:1325-9.

[103] Toshimori H, Toshimori K, Ōura C, et al. *Immunohistochemical study of the atrial natriuretic peptides in the embryonic, fetal and neonatal rat heart*. Cell Tissue Res 1987;248:627-33.

[104] Langub MC, Watson RE, Herman J.P. *Distribution of natriuretic peptide precursor mRNAs in the rat brain*. J Comp Neurol 1995;356:183-99.

[105] Houweling AC, Somi S, Van den Hoff MJB, et al. *Developmental pattern of ANF gene expression reveals a strict localization of cardiac chamber formation in chicken*. Anat Rec 2002;266:93-102.

[106] Firulli AB, Mcfadden DG, Lin Q, et al. *Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1*. Nature Genet 1998;18:266-75.

[107] Gutkowska J, Antunes-Rodrigues J, McCann SM. *Atrial natriuretic peptide in brain and pituitary gland.* Physiol Rev 1997;77:465-515.

[108] von Harsdorf R, Edwards JG, Shen YT, et al. *Identification of a cis-acting regulatory element conferring inducibility of*

the atrial natriuretic factor gene in acute pressure overload. J Clin Invest 1997;100:1294-304.

[109] Tietze F. *Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: application to mammalian blood and other tissues*. Anal Biochem 1969;27:502-522.

[110] Habig WH, Pabst MJ, Jacoby WB. *Glutathione S-transferases.* J Biol Chem 1974;249:7130-9.

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