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The principle and the potential approach to ROS-dependent cytotoxicity by non-pharmaceutical therapies: optimal use of medical gases with antioxidant properties

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Abstract

Regulation of cellular redox balances is important for the homeostasis of human health. Thus, many important human diseases, such as inflammation, diabetes, glaucoma, cancers, ischemia and neurodegenerative diseases, have been investigated in the field of reactive oxygen species (ROS) and oxidative stress. To overcome the harmful effect of oxidative stress and ROS, one can directly eliminate them by medical gases such as carbon monoxide (CO), hydrogen sulphide (H_2S), and molecular hydrogen (H_2), or one can induce ROS-resistant proteins and antioxidant enzymes to antagonize oxidative stresses. This article reviews the molecular mechanisms how these medical gasses work as antioxidants, and how ROS resistant proteins are produced in the physiological context. Targeted therapeutic modalities to scavenge or prevent ROS might be applied in the prevention and treatment of ROS-related diseases in the near future.

Introduction

Reactive oxygen species (ROS) play an important role in aging and life-style dependent diseases such as cancer, stroke and diabetes. Recently hydrogen gas, C60 fullerene, and functional nano-particles were acknowledged as selective anti-oxidants with tremendous application potential [1, 2]. In addition, electro-magnetic waves including different spectrum of light, modulate ROS production for the treatment of several diseases. Further, the most attractive new concept in the field of oxidative stress is the artificial production of ROS-resistant proteins, which was found in long-lived (28 years) naked mole rats [3], suggesting how ROS-related redox signaling could be modified for medical purposes [4]. In the following sections, how ROS are produced, how ROS affect cellular functions, and how ROS are eliminated in living cells will be discussed. Targeting pathways of redox signaling, by ROS in living cells is one of the most rational options to overcome or prevent aging-related diseases.

In order to elucidate ROS-related diseases and problems, three sections will be shown as followings,

I. How ROS are generated in living cells.

II. How ROS are removed from living cells.

III. How ROS resistant proteins are made artificially.

In part I, the detail of the mechanism how light and radiation can generate ROS are discussed. Also, we discuss several metal ions which are involved in several steps of redox signaling in the generation of ROS from oxygen molecule. In part II, we propose several medical gases, including carbon monoxide (CO), hydrogen sulphide (H_2S), and molecular hydrogen (H_2), to counteract ROS. Of note, these gases share some common

factors and signaling molecules in ROS buffering, such as superoxide dismutase (SOD), heme oxidase-1 (HO-1). At first, we discuss the role of key antioxidant enzymes such as SOD, catalase, and glutathione peroxidase in this process. In the part III, ROS-resistant proteins and one of the key redox signaling cascade in living cells will be discussed.

I. Production of ROS in living tissue by light and radiation

ROS and a related term "oxidative stress" were known as a paradigm to describe as an imbalance between oxidants and antioxidants leading to a disruption of redox signaling, resulting in molecular and cellular damages [5]. Accordingly, generation of ROS may contribute to a condition of "oxidative stress" [6]. ROS may also indicate the generic name of singlet oxygen ($^1O_2$), super oxide ($\bullet O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (•OH).

When a photosensitizer (PS), either exogenous or endogenous, is exposed to light, PS will absorb the specific wave length of light and will be excited to singlet state from the ground state. Due to the energy flow, the singlet state will return to the ground state shortly after heat production. Alternatively, it may shift to triplet state, which is more slowly than to singlet state, via the intersystem crossing [7]. The triplet state is more

stable than the singlet state. Eventually, the energy stored at the triple state can decay gradually to ground state via two independent pathways; type I reaction and type II reaction (Fig. 1).

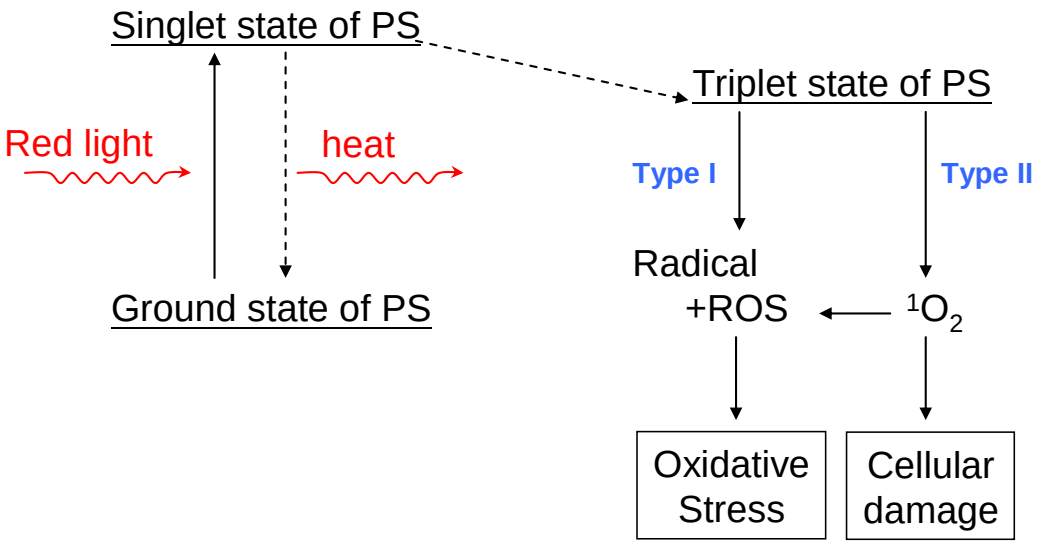

Fig. 1 Excitation of photosensitizer (PS) by red light to develop ROS and singlet oxygen

Type I reaction generates free radicals, which make oxidative stress together with ROS derived from singlet oxygen produced by Type II reaction [8]. Type II reaction is predominant when cells are irradiated with red light [9, 10]. Oxidative stress can result from several other biochemical reactions, including nitrosation [10]. We can classify effect of oxidative stress as a result of modification, such as

nitrosation [11], carbonylation [12], disulfide bond formation [13] and glutathiolation [14]. In addition to those, the effect of oxidative stress on biochemical and physiological effect for cellular dysfunction is collapse of stem cell regulation [15], promotion of cyclophilin A from vascular smooth muscle, activation of transient receptor potential (TRP) channel [16], involvement in amyotrophic lateral sclerosis (ALS) [17]. Also effect of oxidative stress on biological function related to apoptosis is induction of carcinogenesis, activation of MAP kinase, pathway [19], externalization of phosphatidylserine (PS) [20, 21]. Suppression of caspase activity [22], DNA damage [23], induction of mitochondrial membrane permeation [24]

I-1. Photosensitizer as ROS generator

As depicted in Fig. 1, when photo-sensitizer is illuminated by light, the sensitizer can shift to high density triplet state if it has heavy metal ion. This triple state relaxed to lower state with the emission of phosphorescence and making radicals. Generally it is called photochemicals. The most popular reaction is cis-trans isomerization, which is caused when light is illuminated to C-C double bond. This reaction has been established in the retinal rhodopsin [25], β-carotenoide [26], chlorophyl and hemoglobin [27, 28].

Among them, hemoglobin, especially heme (Fe), is an important component of cytochrome. Heme is also required for the synthesis of enzymes other than cytochromes, as well as myoglobin and hemoglobin. Eventually, iron can play an important role in ROS development and in the increase of cytotoxicity, both of which is shown in a cell culture study where cells were incubated with iron either before or after light irradiation [29]. As expected, iron given before light irradiation resulted in less lethality but adding iron after irradiation increased lethality [30]. This is because chelating iron before light illumination results in less photo-sensitizer accumulation [31].

Interaction of human cells with ultra fine (nano-) particles can cause the formation of ROS, including super oxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen [32]. While some types of nano- particle made by carbon, metal and metal oxide act as PS, causing production of both singlet oxygen and super oxide from ground state of oxygen gas molecules under the influence of light, the photochemical reactivity of nanoparticles is regarded of little relevance for the interaction with tissues that are not exposed to daylight. Also transition metal ions may be released in the vicinity of nanomaterials, partly derived from particle impurities, catalyzing Fenton type reactions [33, 34]. In addition, the physical interactions of

particles with subcellular structures involved in the catalysis of redox reactions may modulate generation of ROS. Hypothetical targets of the nanoparticles might involve plasma membrane, mitochondria and endoplasmic reticulum. An interaction of nanoparticle with mitochondrial structure may affect directly to electron flow and leakage from the inner membrane of mitochondria. The interaction of nanoparticle with endoplasmic reticulum (ER) may cause dysregulation of intracellular Ca^{2+} ion level, which in terms activates Ca^{2+}/calmodulin-dependent protein kinases II (CaMKII) and eNOS and nNOS, resulting in production of nitric oxide.

I-2. Effect of UV light for redox signaling

UV radiation is divided into three defined regions of wavelength, including UVA (320-400 nm), UVB (280-320 nm) and UVC (less than 280 nm). Among them UVA penetrate skin most deeply and reach to subepidermal layers [35, 36]. It is well known that UVA is attributed to photosensitized generation of singlet oxygen. The singlet oxygen represents an electrically excited state of O_2, singlet oxygen [37, 38]. Although it is not a radical, singlet oxygen is more reactive than ground state of molecular oxygen due to the "spin-forbidden" rule. Example of such UV photosensitizer includesporphyrins, flavins and certain quinines [39, 40]. This principle of the sensitized

generation of singlet oxygen is exploited medically in photodynamic therapy (PDT), where the sensitizers are administrated and accumulated in target areas prior to irradiation in order to kill the respective cells [41]. How important the singlet oxygen is? Singlet oxygen is electrically excited state of dioxygen, and it has higher energy than the triplet state of O_2, which is generally found in air. The singlet oxygen has high affinity with double bond of fatty acid. As a consequence, it has higher reactivity than triplet O_2. The singlet oxygen can also react with a wide range of biomolecules, including DNA, proteins and lipids.

Higher energy UVB and UVC are not available as UVA for clinical use. UVB is effective to decomposition of ozone (O_3), while UVC is effective for formation of ozone from atomic and molecular oxygen. Without doubt, ozone is another highly reactive form of oxygen and plays a role as oxidative stressor but is probably not formed in human body. Some recent reports claimed that ozone is formed in neutrophils [42-44], but no reports were followed subsequently. Lipid peroxidation is also an important factor to cause damage to living cells. When UV is irradiated to lipid molecules, hydrogen molecule in double bond in LH (unsaturated fatty acids) is cut off and L• (lipid radical) is formed. When O_2 molecules are exist nearby, L• will be LOO•, which will make peroxide radicals (LOOH), activating membrane enzyme such as PLC and

PLA2 [45-48].

I-3. ROS Production by radiation

The major component of living cell is H_2O. When low energy radiation enters into the living cell, G values obtained by low LET radiation is listed as shown in Table 1. LET is defined as energy loss/ cm path length in the water. Gamma ray and β-ray has low LET, while α-ray, proton and heavy ion has high LET [49-51].

Table 1 G values of 1st productions from H_2O by radiation	
Hydroxy radical (• OH)	2.8
Hydrated electron (e^-_{aq})	2.8
Hydrogen (H_2)	0.45
Hydrogen peroxide (H_2O_2)	0.75

In Table 1, G value is defined as number of product when material absorbed 100 eV of radiation.

As shown in Table 1, e^-_{aq} is very specific for ROS production by radiation. The e^-_{aq} is formed by;

$e^- + nH_2O = e^-_{aq}$ (hydrated electron)

and,

$H_2O \rightarrow H_2O\bullet \rightarrow H^- + \bullet OH$

When O_2 is present, H^- and e^-_{aq} make $\bullet O_2^-$ (super oxide) and hydro-per-oxyradical

($HO_2\bullet$). Thus radiation can produce super oxide very effectively in the living tissue. This is called “oxygen effect”. Accordingly, enhancement of radiation effect with oxygen is defined as,

OER (Oxygen enhancement ratio) = Effect without O_2/ Effect with O_2

Usually, observed value of OER is about 2.5 ~ 3.0.

Subcellular localization of ROS production is crucial in the biological processes. It is well known that mitochondria generate ROS largely within the cell. If we introduce artificial lipophilic photosensitizer [52], such as rose bengal, riboflavin, methylene blue and various porphrins, into the cell, it will be preferentially accumulated in the lysosomes [53], mitochondrial membrane [54], Golgi apparatus [55], endo-plasmic reticulum, and plasma membrane.

I-4. Redox signaling and metal ions in the living tissue

Oxygen molecules present in living tissues will become super oxide after several steps of redox signaling eventually. The super oxide will be eventually transformed to H_2O via H_2O_2 and $\bullet OH$. This signaling pathway of ROS production is shown in Fig. 2.

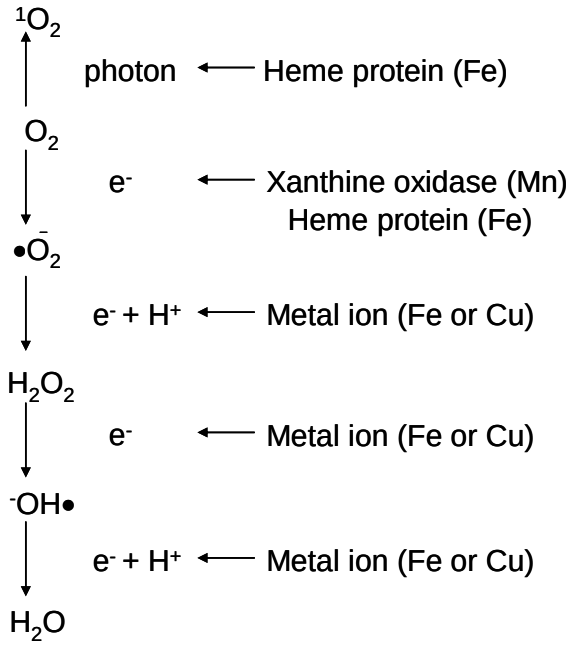

Fig. 2 Redox signaling pathway in the cells in the presence of metal ions.

There are a lot of metals or metallic ions in the human body. In the order of amount in the human body is; Fe > Zn> Cu> Mn> Se> As> Cr> Mo> Co > V> Ni complexes. Many of these complexes contain transition metal ions and are redox active such as cytochrome c. Others take advantage of ligand exchange properties of metal complex (e.g. hemoglobin, myoglobin). For ROS production, Mn (manganese), Fe (ion) and Cu (copper) are major [56]. For ROS removal, Zn (zinc) is important in addition to Mn, Fe and Cu for SOD activation [57]. Se (selenium) is also important for elimination of H_2O_2 by activation of peroxidase with Fe and Mn [58].

Here we discuss As (arsenic) in this paragraph especially, because it is not well understand in the redox signaling. Chronic intake of As was associated with increased risk of cancer, diabetes, developmental vascular diseases and reproduction, all which may result from ROS imbalances. Furthermore, direct evidence that As can cause oxidative damages to DNA and protein was reported recently [59, 60]. Dermatologic toxicities due to As exposure are well documented and include arsenical keratosis, carcinoma in situ, invasive skin cancers, and pigmentary abnormalities [61-63]. In addition to general ROS, nitric oxide (•NO) and peroxynitrite ($ONOO^-$) are referred to as reactive nitrogen species (RNS). Exposure of human keratinocytes to As leads to formation of $\bullet O2^-$ and H_2O_2 [63]. Different from other metal ions listed above, As is a semi-metal or a metalloid with two biologically important chemical state, As (V) and As (III), as the oxyacids (H_3AsO_4) or arsenic trioxide (As_2O_3). The other aspect of As chemistry relevant to biological activity is that As (III) behaves as a soft metal ion, forming strong bonds with thiolates of cysteine residues [64] and the imidazolium nitrogen of histidine residues [65]. It is also not clearly determined yet how As enters into mammalian cells. Recently we have discovered that As selectively reduced IP_3-induced Ca release (unpublished data). It is reasonable to assume that As can enter into the cell via aquaporin channel and react with cystein residues, although cellular

damage by ROS was expected.

II. How ROS are removed from living cells: the elimination of ROS

Next, we discuss how to eliminate ROS in the living tissues by several kinds of enzyme and coenzyme. We will also discuss the minerals that act as activators of the enzymes.

II-1. Super oxide dismutase for superoxide anion radicals

As described so far ROS is collective term of superoxide radical anion ($\bullet O^{2-}$), the hydroxyl radical ($OH\bullet$) and peroxyl radicals ($ROO\bullet$), the most important molecule in the redox signaling, is H_2O_2 as shown in Fig. 2. The fate of most ROS is dismutation to H_2O_2 by both enzymatic and non-enzymatic reactions. Thus it is not surprising that proteins known to react with super oxide are guanylate cyclase [66], ribonucleotide reductase [67], phosphor-protein phosphatase 2B [68].

Catalyzed by SOD, super oxide radical anion makes H_2O_2.

$$\bullet O_2^- + \bullet O_2^- = H_2O_2 + O_2$$

This is an important reaction to protect against cell damages, because half life of super oxide is very short. In humans, SOD occurs in three forms; two of them, SOD1 and SOD3, contain Zn and Cu, while SOD2 contains Mn. SOD1 is present in cytosol, SOD2

is in mitochondria and SOD3 exist outside the cell. However, H_2O_2 is also toxic, because H_2O_2 act as a redox signal eitherthrough redox coupling directly with amino acid residues or indirectly through cellular mediators, such as the thioredoxin and glutathione couples [69]. In some cases, the products of these reactions inhibit the protein functional [70, 71]. Therefore H_2O_2 must be further decomposed by catalase as;

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

and by glutathione peroxidase as;

$$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$$

, where GSH is oxidized to its disulfide GSSG in the reaction.

II-2. Reducing ROS by medical gases

The diverse physiological actions of the ‘‘medical gases’’ such as CO, NO, and H_2S, as well as H_2, have drown attentions. These gases play important roles as cellular signaling molecules. The difficulty in measuring local gas concentrations obscures detailed mechanisms whereby gases exert their actions. Although many questions remain unanswered, some potential mechanisms are summarized as below.

Carbon Monoxide (CO)

CO is a diatomic molecule, and is soluble in aqueous media and organic solvents [72]. CO is relatively stable and has no unpaired electron whereas nitric oxide (•NO)

has one unpaired electron. Not only from exogenous environmental exposure but also from endogenous production during the heme metabolism are major source of CO from primitive prokaryotes to humans [72, 73]. Recent studies have revealed that CO serves as an intrinsic signaling molecule and shows anti-inflammatory and anti-apoptotic effects (Fig. 3). Therapeutic effect of CO is observed in several pathological disease models such as sepsis [74, 75], asthma [76], oxidative-lung injury [77, 78], bleomycin-induced pulmonary fibrosis [79], myocardial infarction [80, 81], hypertension [82-85], atherosclerosis [86, 87], ischemia/reperfusion [88-91], and liver/lung transplantation [92, 93].

Endogenous production of CO is highly associated with heme oxygenases (HO) activity which induces enzymatic degradation of heme. HO breaks the alpha-methene carbon bond of the porphyrin ring with the help from NADPH and molecular O_2 in a reaction that releases equimolar amount of biliverdin, iron, and CO [94, 95]. Of two major isoforms HO-1 and HO-2, HO-1 is inducible whereas HO-2 is constitutively expressed [96, 97]. In an oxidative stress condition, HO-1, which is also named as HSP 32, is induced by several transcription factors including NF-E2-related factor-2 (Nrf2), which binds antioxidant response elements (ARE) [98, 99]. Other transcription elements are also associated with the transcriptional induction of HO-1, including cAMP

response element, TPA response element, and Maf binding site, which are similar to stress-response element [100]. The expression level of HO-1 itself is also induced by CO exposure via nitric oxide (•NO) in bovine pulmonary artery endothelial cells culture [101].

HO can catalyze the first and rate-limiting step in the oxidative degradation of heme to form the open-chain tetrapyrrole biliverdin-IXa (BV) [102, 103]. BV is subsequently converted to bilirubin-IXa (BR) via an NAD(P)H-dependent biliverdin reductases [104]. Both BV and BR can act as potent antioxidants [105-107]. Both BV and BR serve as physiological antioxidants. BR acts as an electron donor, and thereby inhibits lipid peroxidation by chain breaking antioxidant effect [108]. On the other hands, BV reacts with peroxyl radicals, and form carbon-centered radical (or radicals) by the addition of an LOO- to the pigment (LOO• + BV → LOO-BV•) [108]. This radical-trapping activity of BV is relatively higher than BR.

Endogenous CO, which is produced by heme degradation, induces ROS-dependent signaling transduction in the antioxidant defense enzyme systems, including the mitochondrial superoxide dismutase (SOD2) and HO-1 itself [101]. The source of ROS, especially super oxide, is mainly mitochondrial complex III [109]. CO binds at cytochrome c oxidase, which leads to the slowing the transfer rate of electrons to

molecular O_2. This delay in electron transfer make the upstream carriers to more reduced state and fill the Q cycle electron pool, leading to increases in O_2 concentration. MnSOD converts $\bullet O_2^-$ to H_2O_2, and CO-mediated mitochondrial H_2O_2 serves as a signaling molecule to mitochondrial biogenesis. The key transcription factor of mitochondrial biogenesis is nuclear respiratory factor-1 (NRF-1), which is functionally regulated by Akt [110]. Mitochondrial H_2O_2 inactivates counter-regulatory phosphatases such as PTEN and PTP1B, which leads to PI3K-induced unopposed activity of Akt [111-114]. NRF-1 binds to mitochondrial transcription factor-A promoter (*Tfam* promoter). Transcriptional induction of Tfam is essential for mitochondrial DNA transcription and replication [95, 115]. The mitochondrial biogenesis can maintain the mitochondrial number, volume density, and function, which allows the cells to show their adaptive response against mitochondrial damages.

The anti-inflammatory and anti-apoptotic effects of CO are mediated by p38 mitogen-activated protein kinase (MAPK) signaling, which acts in response to physical and chemical stress inducers including oxidative stress, UV, ischemia, and pro-inflammatory cytokines [116]. Activation of p38 also mediates the induction of heat shock protein 70 via its transcriptional factor, heat shock factor-1, leading to the cytoprotective effects [117]. (See Fig. 3)

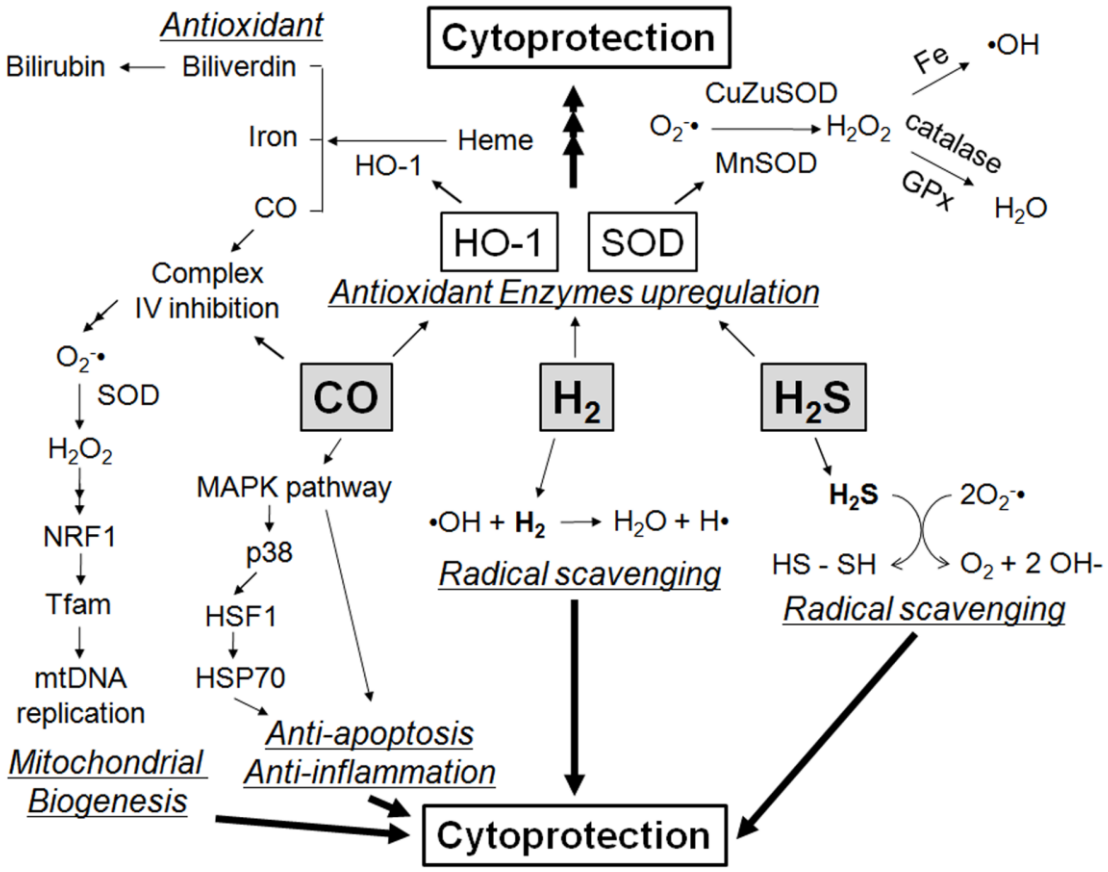

Fig. 3 Schematic mechanisms for cytoprotection induced by medical gases

Hydrogen sulphide (H_2S)

Hydrogen sulphide (H_2S) is a flammable, water-soluble gas with a smell of rotten eggs. Although it is known as a toxic gas and as an environmental hazard, the role of H_2S in tissue has gained an interest with its cytoprotective effects.H_2S can be

synthesized by endogenous two enzymes, cystathionine β-synthase (CBS), and cystathionine γ-lyase (CSE). These two enzymes are pyridozal-5'-phosphate-dependent, and their substrate, L-cysteine, is derived from nutritional supports or liberated from endogenous proteins or peptides. It is also synthesized from L-methionine, and during this synthesis, homocysteine is produced as its intermediate [118-120]. Although higher level (millimolar) H_2S exposure is cytotoxic (free radical generation, glutathione depletion, intracellular iron increase, mitochondrial cell death signal), lower level (micromolar) H_2S shows cytoprotective (anti-necrotic and anti-apoptotic) effects.

Biochemical analysis have revealed that sulphide shows the direct antioxidant reaction with one- or two-electron molecules (one-electron molecules: $\bullet NO_2$, •OH, $CO_3\bullet^-$, two-electron molecules: peroxynitrite, hydrogen peroxide, hypochlorite, taurine chloramine) as well as other low-molecular-weight thiol molecules such as cysteine and glutathione. Although sulphide is not a preferential target for radicals or oxidants due to its low concentration *in vivo*, it can serve as a direct antioxidant [121, 122].

H_2S induces upregulation of anti-inflammatory and cytoprotective genes, including HO-1 [120, 123]. As mentioned above, enhanced HO-1 activity produces CO, which shows cytoprotective effects such as anti-apoptosis, anti-inflammation, and antioxidant effects. This indicates that H_2S might display cytoprotective effects together with the

induction of CO. In brain endothelial cells, H_2S donor, sodium hydrosulfide (NaHS), protects against methionine-induced oxidative stress via induction of redox-regulated enzymes and proteins such as superoxide dismutase, catalase, apocynin, N-acetyl-l-cysteine, and reduced glutathione [124].

H_2S can open the potassium-operated ATP channel (K_{ATP} channel), which is associated with the distinct pharmacological effects of H_2S [125]. K_{ATP} channel opening leads to the vasodilatory and myocardial preconditioning effects [126-128]. In the isolated organ system, H_2S results in smooth muscle relaxation possibly by endothelium-dependent hyperpolarizing factor-like effects [120]. Because the genetic deletion or pharmacological inhibitor of K_{ATP} channel increases myocardial infarct size even in H_2S-treated animals, precise role of K_{ATP} channel in tissue ischemia remains unsettled. [129-131].

H_2S or its donors, NaHS and sodium sulphide provide cytoprotective actions in several disease models, such as myocardial ischemia, carrageenan-induced paw edema, air pouch inflammation, gastric ulcer, colorectal distension, non-steroidal anti-inflammatory drugs induced gastropathy model, and so on [128, 132-135]. On the other hand, H_2S donor can exacerbate pathological processes in several disease models such as sepsis and stroke. Inhalation of H_2S at high levels causes toxic effects such as a

transient loss of olfaction, pulmonary inflammation, cardiovascular events, neurological toxicity [136-138]. Cardioprotective effects mediated by H_2S is bell-shaped, therefore, exquisite balance must be required in either endogenous or exogenous H_2S derived cytoprotective actions [128]. (See also Fig. 3)

Molecular hydrogen (H_2)

Since the first striking evidence indicating that molecular hydrogen (H_2) acts as an antioxidant and inhalation of H_2-containing gas reduces ischemic injury in brain [1], there have been increasing reports which support therapeutic properties of H_2 against oxidative stress-related diseases and damages in the brain [139, 140], liver [141], intestinal graft [142], myocardial injury [143, 144], and atherosclerosis [145]. H_2 can be taken up by inhalation of H_2-containing gas (H_2 gas) or drinking H_2-containing water (H_2 water). One hour after the start of inhalation of H_2 gas, H_2 can be detectable in blood, at levels of 10 μM in arterial blood [1]. The content of H_2 can be measured even after intake of H_2 water by a catheter, which shows 5 μM in artery calculated after 3 min of H_2 water ingestion [139].

H_2 in drinking water rescued the loss of dopaminergic neurons in MPTP-treated mice [146]. The therapeutic effects of H_2 water against Parkinson's disease model have also been confirmed in another animal model, 6-OHDA-treated rats [147]. 6-OHDA can

cause 8-oxoG, an oxidized form of guanine into DNA and RNA by •OH, leading to mitochondrial dysfunction through oxidative stress [148].

H_2 reduces cytotoxic •OH selectively, whereas the production of other radicals such as superoxide, hydrogen peroxide, nitric oxide are not altered by H_2 [1]. This selectivity was proved by cell-free system, and in particular, the preference of scavenging of •OH rather than superoxide was confirmed in PC12 cell culture system [1]. According to Setsukinai et al. [149], both •OH and peroxynitrite ($ONOO^-$) were much more reactive than other ROS. This might address why H_2 shows selective reaction with only the strongest radicals both in the cell-free system and in PC12 cells.

Notably, •OH overproduction in oxidative and neurotoxic reaction by MPTP leads to lipid peroxidation observed by 4-hydroxynonenal (4-HNE) immunostaining in nigral dopaminergic neurons prior to cellular death. Immunoreactivity of 4-HNE in MPTP-treated mice is increased by three times as much as that in saline-treated mice [146], which is similar to the previous report of 4-HNE protein levels in substantia nigra observed at the same period after MPTP administration using HPLC [150]. H_2 water significantly reduces the formation of 4-HNE in dopaminergic neurons in the substantia nigra to the level of control [146]. On the other hand, the increase in superoxide, which is detectable by administration of dihydroethidine (DHE) intravenously, was not

significantly reduced by H_2 water [146]. Although H_2 reduces the production of superoxide in brain slices in hypoxia/reperfusion injury [151], H_2 water might show a preferential reduction of •OH during the protection of dopaminergic neurons.

H_2 water significantly reduces the accumulation of 8-oxoG in striatum after MPTP administration [146]. As mentioned above, 8-oxoG, an oxidized form of guanine into DNA and RNA by •OH, accumulates both in mitochondria and in nucleus; their nomenclature are mt8oxoG and nu8oxoG, respectively. Mt8oxoG accumulates in striatum which are rich in mitochondria in nerve terminal of dopaminergic neurons projected from the substantia nigra. Although nu8oxoG was not detected in nigral cell nucleus, H_2 water might prevent the mt8oxoG-induced cellular apoptotic signals, not just reduce •OH in dopaminergic nerve terminal.

H_2 was effective when it was inhaled during reperfusion; when H_2 was inhaled just during ischemia (not in the reperfusion stage), infarct volume was not significantly decreased [1]. It was shown that H_2 in the brain decreased immediately after stopping inhalation and completely disappeared within 10 minutes [146], indicating that the effect of H_2 can be observed only during the period when the oxidative insults occur. According to the previous report [139], H_2 could be detected in the blood 3 minutes after administration of H_2 water into the stomach. However, our unpublished data

showed that the half-life of H_2 in the muscle in rats was approximately 20 minutes after the administration of H_2 gas. Taking these reports into consideration, H_2 in the brain and other tissues does not stay long enough to exert its ability as an antioxidant to ROS directly. Therefore, it is unlikely that direct reaction of H_2 itself with ROS plays a major role in the neuroprotection, especially by H_2 in drinking water, even though H_2 itself has the ability to reduce •OH preferentially. In accordance with this hypothesis, drinking H_2 water increases urinary antioxidant enzyme, superoxide dismutase (SOD) [152], like polyphenols, as an endogenous defensive system against ROS (especially superoxide)-mediated cellular damage. Although it takes eight weeks for significant increase of SOD in human, H_2 is able to alter the expression level of urinary antioxidant enzyme. It was also reported that H_2 water increases total bilirubin for four to eight weeks when compared to baseline. Bilirubin is produced by the catalytic reaction of HO-1, and degradation of heme generates bilirubin as well as carbon monoxide and free iron. The increase of HO-1 expression may result from the response to oxidative stress, which is also characterized as a phase II antioxidant and positively regulated by several stress-responsive transcriptional factors [100]. Therefore, taking these observations into consideration, there seems to be other mechanisms for protective effect of H_2 in drinking water apart from inhalation. It is possible that drinking of H_2 water has not

only the ability to reduce cytotoxic radicals, but also novel mechanisms which are related to anti-oxidative defense system. These cytoprotective mechanisms of H_2 are quite similar to those of other medical gases (gasotransmitters), CO and H_2S, as we have discussed above. In conclusion, H_2 could be another gas mediator which shows therapeutic effects like CO and H_2S (Fig. 3).

III. How ROS resistant proteins are made artificially: a different way to overcome ROS toxicity

As was described above, ROS toxicity was overcome by activation of SOD or by medical gases. Another efficient way to overcome ROS would be producing ROS-resistant proteins. There was a cohort study in Taiwan showing that low-dose ionizing irradiation for many years was turned out to treat and cure many illnesses, including cancer [153]. It is serendipitous application of low-dose radiation. Another interest comes from the fact obtained from “naked mole rat”, which is well adapted for the limited availability with oxygen due to high affinity for oxygen of its hemoglobin. It has very low respiration and metabolic rate for animal of its size. It is extraordinarily long lived for a rodent of its size (up to 28 years) due to prevention of oxidative damage. According to Perez, et al. [3], the longevity of the rat may attribute to a very stable

structure of protein. This stability may be ascribed to high level of cysteine in their proteins for shielding them from oxidation. Unexpectedly, Western blot analysis of Hsp70 and Hsc70 levels in liver homogenates showed no age-related difference in the rat. This is because heat shock protein is produced by differential heat stimulation or transient ROS stimulations, while ROS resistant proteins produced in the rats without any kinds of transient stimulations. Therefore it is reasonable to assume that inherent cysteine (Cys) content may serve as a determination of longevity in the rat, while ROS-resistant protein may be also produced by transient ROS exposure repeatedly.

III-1. ROS resistant protein formation by low dose radiation

As described in I-1, radiation including X-ray is most effective means to produce ROS. Therefore it is likely that ROS resistant protein can be produced with high efficiency. In the case of heat shock protein, as a good example, it is already known that transient change in the external media from regular extracellular temperature to 40°C-43°C will be a trigger for whole process.

The level of structural organization of globular proteins is;

Primary (amino acid) sequence → secondary sequence →

→supersecondary sequence → domain → globular protein→aggregation

The constitution determines the system of covalent bonds in the protein macromolecule

and includes information about disulfide bridges that form spontaneously during protein syntheses on ribosome. The secondary structure is a regular geometric figure of chain created by H bonding between the C-O and N-H group of peptide bonds. Further folding of polypeptide chains result in the formation of tertiary structures. They are produced by long range contacts within the chain and stabilized quaternary structure is an organization of protein subunits or two or more independent polypeptide chains.

The spontaneous act of protein folding is remarkable in that the complex motion of a protein structural element. This process results from thermally activated Brownian motion and resembles a phase transition, such as coupling. Consequently the net stabilization of the native state of protein conformation results from a balance of large force that favors both folding and unfolding. The net free energy of folding is 10 kcal/mol for hypothetical protein. By the combined effect of hydrophobicity of some side chains of amino acids and the structure of water side chain of non-polar amino acid residues is to locate in the interior of a protein. The order of hydrophobicity and hydrophilicity for the amino acids is:

Hydrophobic: Phe> Ala > Val > Leu > Cys

Hydrophilic: Tyr > Ser > Asp > Glu > Asn > Gln > Arg

Consequently, hydrophobic side chain coalesces in the interior of proteins. Much of the

free energy of protein folding is entropic. It is possible protein unfolding will be happened when conformational entropy was increased. This process was well confirmed by us recently using deuteron as a probe [155]. This can be denominated as two stable state theory. Therefore it is natural that heat shock signal is triggered by unfolding of proteins which is formed by hydrophobic collapse, intramolecular H bonding and van der Waals interaction.

When ROS attacks inside the cells, hydrophilic amino acids are released in the cytosol then tertial structure of the functional protein are destroyed and Ser, Asp, Glu and Arg (well known neuro-transmitter) can move just like second messengers and activate synthesis of ROS-resistant proteins.

III-2. Overview of non-drug therapy according to the thermodynamic principle for redox signaling

We are living by consumption of Gibbs free energy (F), which is expressed as,

$$dF = dU - TdS$$

, where dU is the change in the internal energy of the system (e.g. energy owing to the formation of ATP from ADP, or growth, etc), T is absolute temperature, dS is the entropy change (e.g. the rate of heat flow to the environment, passive diffusion and heat production), dF is sum of the free energy introduced into the system (e.g. radiation,

oxidation and energy influx) and the work that may have to be done (e.g. muscular work, diffusion against concentration gradient).

Most of the processes in our living body system are non-voluntary biochemical reactions, such as protein synthesis from amino acids. Energy source in living cells to synthesize protein is supplied from ATP. When ATP is converted into ADP, Gibbs energy (G) is released by ATP decomposition, associated with increased entropy (S). The released G is used to connect amino acids to make protein. For example connection of 2 amino acids, decomposition of 3 ATP molecules is needed.

Attention must be paid that $dS_l < 0$ and $dS_d > 0$, where subscript "l" means living organism and "d" means death of living organism. $S_d > 0$ coincides with the halt of feedback loop. Accordingly aging or senescence means S_l will increase gradually to positive values. What is the trigger signal for changing S_l from negative to positive?
It is well known that irradiation, local heating and oxidation reaction resulted sequential rearrangements as described in section I. These changes lead to the degeneration of bio-molecules which then undergo further replication. Tertiary and quaternary structures of proteins, for example, are easily altered by thermal vibration. The denaturation of protein is observed at temperature above 42°C. UV and X-ray irradiation kill cells or make errors in replication and translation, which may lead to cancer. Ionizing radiation

can penetrate into the tissue much deeper and cause the decomposition of molecules. Finally, we concluded that removing toxic ROS and promotion of damage repair by producing chaperon are the most rational ways to survive and live a longer time. In Fig. 4, bird's-eye view of non-drug therapy for aging and health medical science is shown.

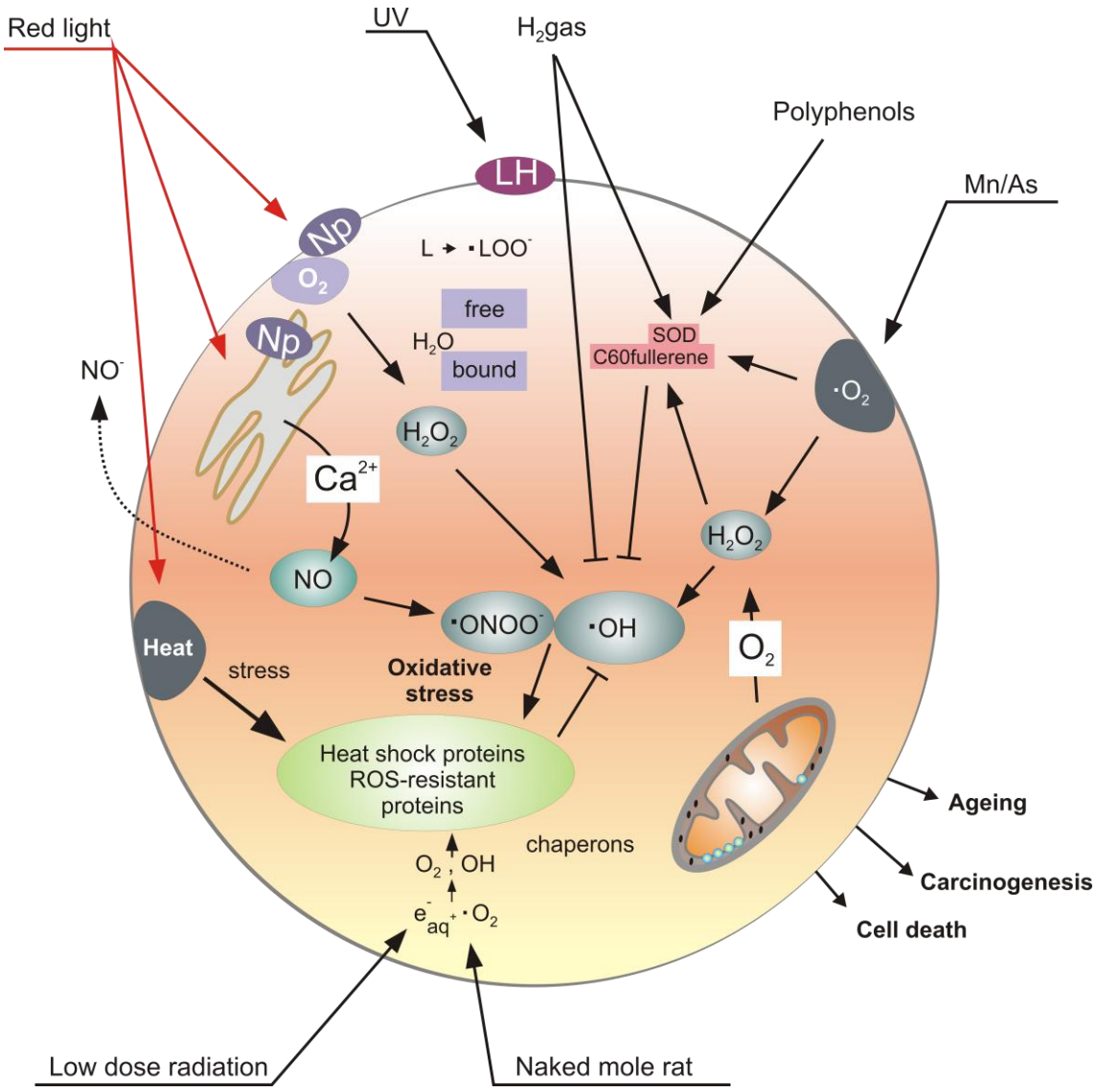

Fig. 4 Bird’s-eye view of redox signaling in the living cell

Clockwise from the left bottom, low dose radiation, red light and UV are for ROS production; Hydrogen gas, minerals such as manganese and arsenic are for elimination of ROS-induced damage. Naked mole rat model may give us a novel idea how to make ROS-resistant proteins for longevity. Low dose ionizing radiation may also be a serendipity to produce chaperons as ROS-resistant proteins. LH: unsaturated fatty acids, Np: nanoparticle.

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