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XANTHINE DEHYDROGENASE/XANTHINE OXIDASE AND OXIDATIVE STRESS

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ABSTRACT

Xanthine dehydrogenase (XDH) and xanthine oxidase (XOD) are single-gene products that exist in separate but interconvertible forms. XOD utilizes hypoxanthine or xanthine as a substrate and O_2 as a cofactor to produce superoxide ($\cdot O_2^-$) and uric acid. XDH acts on these same substrates but utilizes NAD as a cofactor to produce NADH instead of $\cdot O_2^-$ and uric acid. XOD has been proposed as a source of oxygen radicals in polymorphonuclear, endothelial, epithelial, and connective tissue cells. However, several questions remain about the physiological significance and functions of XOD on aging and oxidative stress. XOD is reported to play an important role in cellular oxidative status, detoxification of aldehydes, oxidative injury in ischemia-reperfusion, and neutrophil mediation. For example, XOD may serve as a messenger or mediator in the activation of neutrophil, T cell, cytokines, or transcription in defense mechanisms rather than as a free radical generator of tissue damage. Emerging evidence on the synergistic interactions of $\cdot O_2^-$, a toxic product of XOD and nitric oxide, may be another illustration of XOD involvement in tissue injury and cytotoxicity in an emergent condition such as ischemia or inflammation.

KEY WORDS

Xanthine dehydrogenase, Xanthine oxidase, Aging, Oxidative stress

INTRODUCTION

Biological aging processes are time-dependent, deteriorative changes that occur in all organisms. According to the oxidative stress theory of aging, the aging process is a consequence of deleterious effects of the reactive oxygen species produced during the course of cellular metabolism (1). In recent years, free radicals and other reactive intermediates produced in normal metabolic processes have been implicated in the pathogenic mechanism of a wide range of diseases, including inflammatory diseases, cancer, atherosclerosis, and liver injury. One particular type of tissue injury from free radicals is reoxygenation injury following reperfusion of ischemic tissues (2-8). Metabolic free radical sources include XOD, prostaglandin (PG), hydroperoxidase, NADPH dehydrogenase, mitochondrial electron transport, and peroxisomal enzymes such as amino acid oxidase and fatty acyl CoA oxidase (9,10).

Xanthine oxidoreductase (XDH/XOD) exists in separate but interconvertible forms: XDH (EC 1.1.1.204; xanthine: NAD oxidoreductase) and XOD (EC 1.1.3.22; xanthine: oxygen oxidoreductase) (11). XOD utilizes hypoxanthine or xanthine as substrate and O_2 as cofactor to produce $\cdot O_2^-$ and uric acid:

$$\text{Hypoxanthine/xanthine} + O_2 \xrightarrow{\text{XOD}} \cdot O_2^- + \text{uric acid} \quad [1]$$

The significance of this reaction is that, under certain pathophysiological conditions, XOD can be a major source of $\cdot O_2^-$ intracellular production. In 1968, Della Corte and Stirpe reported that this mammalian enzyme exists in the cell originally in the form of XDH and is converted to the XOD form by enzyme molecule modification (12). As shown in Figure 1, this conversion occurs either reversibly by the oxidation of sulfhydryl residues or irreversibly by proteolysis (12). In contrast to XOD, the native form, XDH, acts on the same substrates but utilizes NAD as a cofactor, producing NADH instead of $\cdot O_2^-$ and uric acid:

$$\text{Hypoxanthine/xanthine} + \text{NAD} \xrightarrow{\text{XDH}} \text{NADH} + \text{uric acid} \quad [2]$$

XDH/XOD serves as a rate-limiting enzyme to catalyze the nucleic acids found in many animal species. During free radical metabolism and oxidative stress, XOD is a major cellular source of $\cdot O_2^-$, and its role as a causative factor in ischemia/reperfusion damage is well documented. On the other hand, a product of the XDH catalytic reaction, uric acid, has been reported to have a potential physiologic function as a protective agent against oxidative damage of lipids, proteins, and purine (see below) (13). This protection is afforded by the assumed role of uric acid as a potent iron chelator and an excellent scavenger of hydroxyl radical, singlet oxygen, hypochlorous acid, oxoheme oxidants, and hydroperoxyl radicals (14-16). Also, XOD is a known source of oxygen free radicals in polymorphonuclear,

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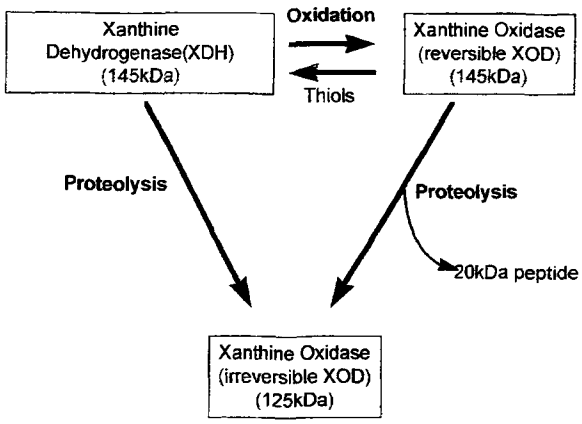

Figure 1. Interconversion of the various forms of XDH/XOD (modified from Della Corte and Stripe, 1972).

endothelial, epithelial, and connective tissue cells. However, several questions remain unanswered about the physiological significance and functions of XOD in aging and aged-related oxidative stress. The present review considers basic biochemical properties, roles, conversion mechanisms, and contributions of XDH/XOD in tissue damage.

Interrelationship of XDH and XOD

Basic Biochemistry. With a size of 300 kDa, and composed of two identical, independent subunits, each subunit contains one molybdopterin, two nonidentical Fe_2S_2, centers, and flavin adenine dinucleotide (FAD) (17,18). The molecular weight of each subunit of the native enzyme prepared without proteolysis is 150 kDa (19,20), although various bands (SDS-PAGE) have been detected with different molecular sizes in samples prepared without protection from proteolysis (21). The full amino acid sequence of liver enzymes from human (22), rat (20), mouse (23), chicken (24), and the enzyme from *Drosophila* (25) have been determined from cDNA cloning. Enzymes consist of around 1,330 amino acids, with an approximate 90% homologous sequence to rat, mouse, and human enzymes (20,22,23).

Limited proteolysis with trypsin converts XDH to XOD with concomitant cleaving into three fragments of 20, 40, and 85 kDa. These fragments are dissociated only under denaturation conditions, such as in the presence of high concentrations of guanidine-hydrochloride (20), suggesting a tight association among fragments.

From amino acid sequencing (20) and chemical modification studies (26), the redox centers are assumed located in three domains: the two iron-sulfur centers in the 20 kDa domain, the FAD in the 40 kDa domain, and the molybdopterin in the 85 kDa domain (20). The analysis of the *Drosophila* enzyme provides a similar conclusion (27). Also, by limited proteolysis, under specific digestive conditions, the chicken liver enzyme (24) and the bovine milk enzyme (21) were isolated having 20 kDa and 85 kDa complexes that contain only Fe/S and molybdenum centers, which supports the 40 kDa fragment of the FAD domain as shown in Figures 1 and 2.

XDH can be irreversibly converted to XOD with proteolytic action, or reversibly with sulfhydryl oxidants (9,28,29,30) in the same proteolytic cleavage sites as described in Figure 2. During conversion from XDH to XOD with sulfhydryl oxidant, several oxidized cysteine residues were detected. (See below for details.)

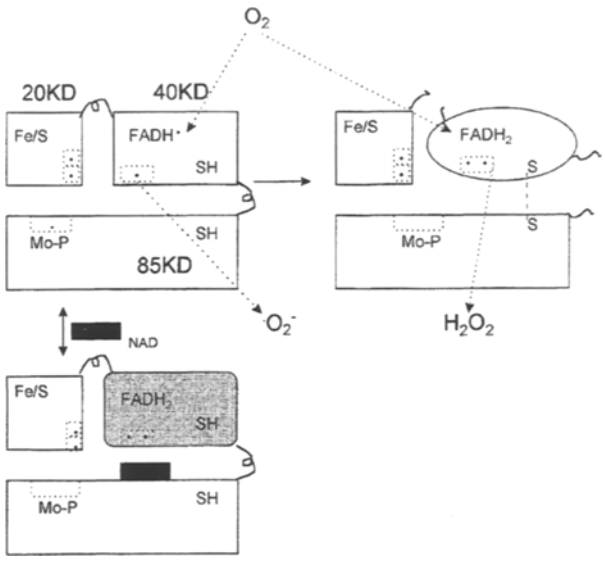

Figure 2. Schematic presentation of XDH/XOD on conformational change by proteolysis, sulfhydryl oxidation or NAD binding involving electron distribution of 4-electron reduced state.

Kinetic Parameters

The differences between XDH and XOD properties are mainly due to the redox properties of their cofactors and their kinetic behavior in steady state. It has been reported that the difference in the condition of sulfhydryl groups of the enzyme brings about drastic changes in the redox and kinetic properties of the enzyme. The conformational change in FAD domain of XDH/XOD causes different reactivity in its cofactors, NAD or O_2. Table 1 shows the kinetic parameters of xanthine-NAD and xanthine-oxygen catalyzed by both types of the enzyme. The K_m values for xanthine are below 5 μM in all the cases studied. The V_{max} value of xanthine-O_2 activity catalyzed by XOD and that of xanthine-NAD activity catalyzed by XDH are close, 1030 and 810 mol/min/mol of FAD, respectively. The V_{max} value of xanthine-O_2 activity catalyzed by XDH is 270 mol/min/mol of FAD, and the K_m value for O_2 is 260 μM, which is about five times as high as that of XOD (31).

Table 1. Kinetic parameters obtained from purified rat liver xanthine oxidizing enzymes (31).

	XDH	XOD
Xanthine–NAD activity		
K_m for xanthine (μM)	1.3	
K_m for NAD (μM)	8.5	
V_{max} (mol/min/FAD)	810.0	
Xanthine–O_2 activity		
K_m for xanthine (μM)	2.8	1.8
K_m for NAD (μM)	260.0	46.0
V_{max} (mol/min/FAD)	270.0	1030.0
Reduction of cytochrome C (Formation of $\cdot O_2^-$ mol/min/mol FAD)	63.0	290.0
Formation of urate (mol/min/mol FAD)	93.0	940.0
$\cdot O_2$/urate (mol/mol)	0.68	0.30

K_m, Michaelis–Menten constant; V_{max}, maximum velocity in Michaelis–Menten equation.

Since XOD catalysis is responsible for intercellular $\cdot O_2^-$ production, it is important to know the kinetic nature of XOD. The $\cdot O_2^-$ formation estimated from the rate of reduction of cytochrome C is shown in Table 1. Without NAD, XOD oxidized 940 mmol of xanthine/min/mol of FAD, and produced 290 mmol of reduced cytochrome C/min/mol of FAD. This indicates that about 30% of the electrons derived from xanthine were transferred to O_2 to form $\cdot O_2^-$ during a steady state turnover (31).

The formation of uric acid by XOD reaction can be estimated by the use of inhibitors because the formation of $\cdot O_2^-$ by XDH can be effectively inhibited by NAD without suppressing XOD. This results in a stochiometric yield of $\cdot O_2^-$ to urate on a ratio of 3:10.

Conversional Status

The schematic diagram in Figure 2 shows the models of 4-electron reduced states and the localization of redox centers suggested by the analysis of the amino acid sequencing and chemical modification studies (32,33). Two iron sulfur centers are associated with the 20 kDa fragment; the NAD and FAD binding sites are associated with the 40 kDa fragment; and the molybdenum is associated with the 85 kDa fragment. Although these cofactors are not directly associated with one another, the distance between them might not be great because the intramolecular electron transfer is very rapid (34). The conformation of the native dehydrogenase is such that FAD is stabilized mostly in the semiquinone state, and reacts slowly with oxygen to form, primarily, superoxide anion (31,35). When the enzyme is modified by proteolysis or sulfhydryl-oxidation, the protein conformation changes particularly around FAD. In this new conformation state, FAD prefers a fully reduced state, which reacts rapidly with oxygen to form hydrogen peroxide. If NAD is present, a conformational change occurs, favoring the fully reduced states of FAD, which react rapidly with NAD (31,35).

Tissue Distribution

Wide distribution of XOD may potentially implicate many physiologic functions. As shown in Table 2, the tissue localization of XDH/XOD activity was investigated in unfixed cryostat sections of various rat tissues by an enzyme histochemical method and by using oxidase forms of XDH/XOD. High activity was found in the epithelial cells from skin, vagina, uterus, penis, liver, oral and nasal cavities, tongue, esophagus, fore-stomach, and small intestine. In addition, activity was observed in the sinusoidal cells of liver and adrenal cortex, endothelial cells of various organs, and connective tissue fibroblasts. At present almost no information is available on the status of XDH/XOD in aged organs or tissues.

Putative Physiological Functions of XDH/XOD

Epithelia-related Functions. On the basis of tissue compartment localization and cellular morphology, endothelial cells and fibroblasts are expected to have high XOD/XDH activity. Interestingly, high enzyme activity is found in the external and internal epithelial cell surfaces of various organs and in the cortical cells of the adrenal gland, as shown in Table 2 (36). In stratified epithelia, the activity is found mainly in the superficial layers, with virtually no activity found in the basal layers.

Recently, XDH/XOD activity was detected biochemically in vaginal epithelial cells and was found to be regulated by hormones (37). In an earlier study, Reiners and Rupp detected enzyme activity in isolated epidermal keratinocytes (38). Moreover, these investigators found that during differentiation keratinocyte subpopulations convert the dehydrogenase form to the oxidase form of XDH/XOD. Various other studies show the induction of cell differentiation by oxygen radicals or hydrogen peroxide (39); therefore, it is possible that superoxide radicals and hydrogen peroxide, produced by the oxidase form of XDH/XOD, are involved in this process.

While another product of XDH/XOD, urate, is a relatively less effective scavenger of $\cdot O_2^-$ and H_2O_2, it is a strong antioxidant of superoxide anion-derived radicals (e.g., $\cdot OH$) (40,41). Differential roles for $\cdot O_2^-$ and H_2O_2 can be maintained when urate is present, and vice versa — an antioxidant function for urate can be maintained in addition to differential roles of $\cdot O_2^-$ and H_2O_2. A minute amount of $\cdot O_2^-$ produced by the xanthine/XOD system has been shown to stimulate DNA synthesis in resting Balb/3T3 fibroblasts and to enhance the proliferation of human fibroblasts (39). The possible physiological conversion of the dehydrogenase form to the oxidase form during differentiation may also play a role in inducing cell proliferation.

Kooij et al. found high XDH/XOD activity in epithelial cells as well as in endothelial cells from various organs (36), leading to suggested functions for XDH/XOD in these cells. Epithelial and endothelial cells are exposed to relatively high oxygen concentrations from the blood

Table 2. Xanthine oxidase activity in rat tissues detected by histochemical method (HM) or by biochemical methods (BM) (36).

Organ or system	HM	BM-p	BM-np
Cardiovascular system			
Heart		d,e,f,g,h[1]	
Endothelial cells	2		
Aorta	0		
Liver		a,b,c,d,e,f,g	
Sinusoidal cells	3		
Hepatocytes (pericentral)	2		
Hepatocytes (periportal)	1		
Urinary system			
Kidney		a,b,c,d,e,f,g	
Interstitium	1		
Brush border (prox. tub.)	1		
Bladder	0		g
Endocrine Organs			
Pancreas		f,g	
Connective Tissue	2		
Thyroid glands			
Connective Tissue	1		
Thymus			
Connective Tissue	1		g
Adrenal gland		g	
Zona glomerulosa	0		
Zona fasciculata	3		
Zona reticulata	3		
Skin (dorsal, abdomen)		b,h	
Epidermis	3		
Connective Tissue	3		
Hematopoietic Organs			
Spleen		a,b,c,d,e,f,g,h	
Red pulp	2		
White pulp			
Marginal sinus	2		
Bone marrow	0		
Knee			
Synovial lining cells	3		
Muscle Tissue		d,g	b
Skeletal muscle			
Connective tissue	2		
Respiratory system			
Nasal cavity			
Epithelium	3		
Connective tissue	3		
Trachea			
Connective tissue	2		
Lung	–		
Alimentary tract			
Oral cavity			
Epithelium			
Basal layer	0		
Intermediate layer	2		
Superficial layer	2		
Connective tissue	2		
Tongue			
Epithelium			
Basal layer	0		
Intermediate layer	2		
Superficial layer	2		
Desquamating layer	3		
Esophagus		g	
Epithelium			
Basal layer	0		
Intermediate layer	2		
Superficial layer	2		
Desquamating layer	3		
Connective tissue	2		
Stomach		b,g,h	
Muscle layer			
Plexus of Auerbach	3		
Fore-stomach			
Epithelium			
Basal layer	0		
Intermediate layer	3		
Superficial layer	3		
Desquamating layer	3		
Connective tissue	2		
Glandilar part			
Connective tissue	2		
Small intestine	3	b,c,e,f,g	
Enterocyte	3		
Goblet cells	3		
Connective tissue			
Colon			
Connective tissue	3		
Caecum			
Connective tissue	3		
Rectum			
Connecive tissue	3		
Brain			
(Cortex and Medulla)			
Cerebellum	0	e,g	b,d
Cerebrum	0		
Female reproductive system			
Vagina			
Epithelium			
Basal layer	0		
Intermediate layer	3		
Superficial layer	3		
Connective tissue	3		
Uterus			
Epithelium	1		
Connective tissue	3		
Male reproductive system			
Testis		g	b
Connective tissue (around seminiferous tubules)	1		
Penis			
Epithelial cells	3		
Connective tissue	3		
Prostate		g	
Connective tissue	1		
Epididymis	–	g	
Seminal vesicle	–		g
Diaphragma	–		g
Mesenterium	–		g

p = present; np = not present; – = not done.

Histochemical activity is scored on a scale of 0 (negative) to 3 (high activity). a. Morgan et al. (1992); b. Westerfeld and Richert (1949); c. Richert and Westerfeld (1949); d. Brunschede and Krooth (1969); e. Ramboer (1969); f. Battelli et al. (1972); g. Hashimoto (1974); h. Krenitsky et al. (1974).

or the environment. And because $\cdot O_2^-$ radicals are produced normally in aerobic cells (42), their production may increase when cells are exposed to high oxygen tension (43). This notion is further envisioned by the finding of high antioxidant enzyme activity in epithelial cells (39), as well as the immunohistochemical localization of superoxide dismutase (SOD) in these cells (44,45).

In this regard, it is interesting to note that XDH/XOD is a rate-limiting step in purine catabolism, produces strong antioxidant urate (40,41,46), and is possibly involved in the antioxidant defenses in saliva (47) and rat lung tissue (48) already reported. Further evidence indicates that urate scavenges oxygen-derived radicals generated in endothelial cells in heart in vivo (49). One major question to be explored is the dual role of XOD, namely the reproduction of $\cdot O_2^-$ and urate.

Oxidative Roles

Recent in vitro and in vivo evidence strongly implicated XOD in the important role of iron absorption in the small intestine and iron mobilization in the liver (50,51). Intestine studies by Tophan and colleagues indicate dietary iron, absorbed almost exclusively in the ferrous state, is oxidized to the ferric state by XOD in the mucosal cell. The oxidative capacity of XOD can be exhibited in other systems as well. For instance, XOD is capable of oxidizing a wide variety of substrates such as pteridenes and other heterocyclic bases, as well as aldehydes.

Aldehydes are among the products of lipid peroxidation; and, of special interest are the 2-alkenal and 4-hydroxy-2-alkenal products arising from peroxidation of poly-unsaturated fatty acid (52). These aldehydic products are biologically active (53), with 4-hydroxy-2-nonenal receiving particular attention for its cytotoxic properties (54,55). The cytotoxicity of trans-alkenals is caused in part, from the reaction of cellular nucleophiles, such as sulphydryl groups, and with electrophilic carbon-carbon double bond (54), leads to the formation of carboxylic acids, effectively diminishing the electrophilicity of the reactive center, and thereby converting into readily detoxificable compounds in vivo (55).

In the liver, XOD catalyzes the oxidation of acetaldehyde to acetate. Kato et al. (56) have recently suggested the role of XOD in ethanol-induced lipid peroxidation.

Urate as an Antioxidant

Bailey et al. reported that the inhibition of XOD increased the susceptibility of liver to reperfusion injury and suggested that XDH/XOD may protect the liver against oxidative injury by forming uric acid in low flow ischemia-reperfusion (57). Tan et al. reported that in human plasma, uric acid is an effective inhibitor in the formation of superoxide and hydrogen peroxide produced by XOD. They suggest that plasma uric acid may play an important physiological role in attenuating the oxidant-mediated tissue damage caused by the XOD released into the circulation during ischemia-reperfusion (58). Furthermore, Radi et al. reported that uric acid behaves as an noncompetitive inhibitor of XOD in the reducing substrate, xanthine (59).

Becker proposed that urate protects against reperfusion damage induced by activated granulocytes-cells, which are known to produce a variety of radicals and oxidants. Urate was shown to prevent oxidative inactivation of endothelial enzymes (cyclooxygenase, angiotensin converting enzyme), and preserve the ability of endothelium to mediate vascular dilation in the face of oxidative stress. Together, Becker's findings suggest a particular relation between the site of urate formation and the need for a biologically potent free radical scavenger and antioxidant (60).

Contribution of XOD to Free Radical Generation and Ischemic Tissue Damage

A Cascade Model. Ischemia-induced tissue injury is now well-recognized as a major factor in the pathogenesis of life-threatening disease (e.g., coronary artery disease and stroke). In the past, the hypothesis that free radicals are critical mediators of postischemic tissue injury has been intensively investigated. This proposal of cascading events is summarized in Figure 3. As a result of a selective proteolysis, NAD^+-reducing XDH is converted to oxygen-reducing XOD. When the tissue is reperfused (i.e. molecular oxygen reintroduced into the tissues), oxygen interacts with hypoxanthine and XOD to produce $\cdot O_2^-$. Once formed, superoxide converts to H_2O_2 and the hydroxyl radical; together, these compounds cause oxidant tissue injury (61).

According to the sequence of events during the ischemic period, ATP is catabolized thereby depleting ATP to hypoxanthine, which accumulates in the tissues. A consequence of this energy depleted state is the increased influx of Ca^{2+} into the cell. The increased intracellular Ca^{2+} then triggers the conversion of XDH to XOD via a protease (62).

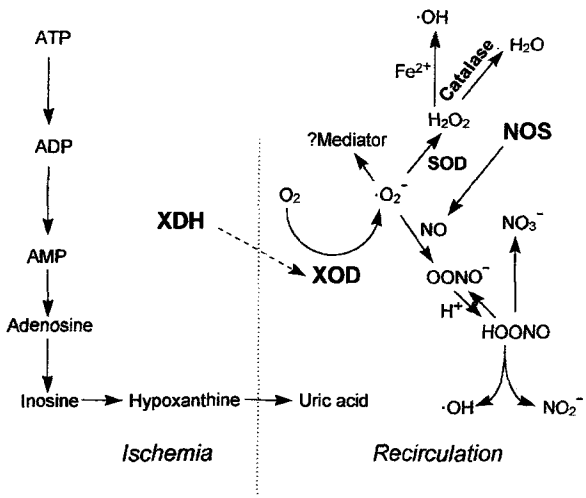

Figure 3. Involvement of xanthine oxidase in ischemic injury. XOD, xanthine oxidase; XDH, xanthine dehydrogenase; SOD, superoxide dismutase; NOS, nitric oxide synthase.

Recently, many studies provide strong support for the involvement of reactive oxygen species in brain injury during cerebral ischemia (63-72). Yet, no clear information is available concerning a precise location or source of these toxic oxygen species. In other organ systems, the enzyme XOD has received considerable attention as a source of free radicals during ischemia and reperfusion (73). This enzyme catalyzes the oxidation of hypoxanthine to xanthine, xanthine to uric acid, and in the process, generates superoxide radicals. XDH, another form of this same enzyme, catalyzes similar reactions, but uses NAD as an electron acceptor and, therefore, does not produce free radicals. In healthy tissue, most of the enzyme exists as XDH. However, XDH is converted to XOD in many ischemic tissues (73-75), and because hypoxanthine accumulates during ischemia (73), reoxygenation may stimulate the production of $\cdot O_2^-$ by XOD.

Although the level of XOD in brain is low compared with other tissues, its activity is enriched in brain microvessels (76). Furthermore, XOD and/or XDH are active in the brain during incomplete ischemia because uric acid accumulates (77-79). Conversion of XDH to XOD during 30 min of global cerebral ischemia has recently been reported (67). Finally, ischemic brain damage is shown to be attenuated either by pretreating animals with XOD and/or the XDH inhibitor, allopurinol, or by feeding animals a tungsten-rich diet, which inactivates XOD (68-79). XOD is a potential source of reduced oxygen that intermediates in the pathogenesis of postischemic tissue injury (70).

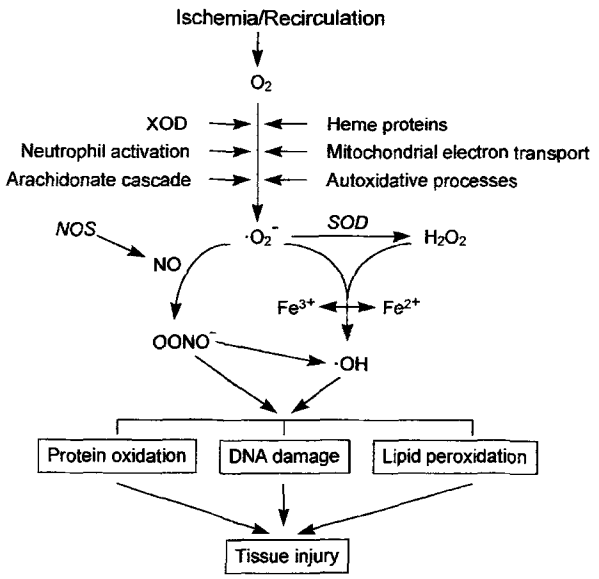

Figure 4. Possible mechanisms of ischemic injury by free radicals. XOD, xanthine oxidase; SOD, superoxide dismutase; NOS, nitric oxide synthase.

Differential Effects

Considerable debate, however, continues regarding the contribution of XOD-derived reactive oxygen metabolites to myocardial damage in humans with coronary artery disease. This controversy arises from the wide interspecies variations in myocardial XOD activity. Specifically, although most models of myocardial infarction have used animal species with relatively high myocardial XOD activity, observations have excluded XOD participation in postischemic myocardial injury in these species (72-76). In parallel treatments with the specific XOD inhibitor, allopurinol, the size of myocardial infarction following ischemia-reperfusion injury generally decreased in rat and dog, but not in rabbit models (72,77-80). To further confuse the issue, XOD activity is reported in both human and rabbit myocardium (81,82). Others have also demonstrated that allopurinol treatment may preserve rabbit myocardial function after ischemia-reperfusion (83,84). Because of this apparent contradiction, it remains unclear whether XOD actually exists or contributes to myocardial reperfusion injury in species such as humans and rabbits. However, Terada et al. proposed that relatively low amounts of XOD activity, similar to levels reported in human myocardium, may contribute to cardiac ischemia-reperfusion injury (85). $\cdot O_2^-$ is also thought to act as a neutrophil chemo-attractant. Therefore, potential sources of free radical production in ischemia, such as those activated by neutrophils, can be considered a contributing mechanism (86,87).

Implication of Nitric Oxide and Other Free Radicals in XDH/XOD Status

Nitric Oxide Radical. Nitric oxide ($NO\cdot$) is very lipophilic, therefore readily diffuses through cellular membranes (88), and can react with other radicals, such as $\cdot O_2^-$. $\cdot O_2^-$ and $NO\cdot$ can react simultaneously with one another to rapidly form peroxinitrite ($ONOO^-$) (89), a highly reactive oxidizing agent, capable of furthering cytotoxicity and causing tissue damage. $ONOO^-$ can oxidize methione residues in proteins and peptides as well as thiols and thioethers (90). This reaction probably occurs in vivo (91) via the following biochemical pathway:

$$NO\cdot + \cdot O_2^- \rightarrow ONOO^-$$
$$ONOO^- + H^+ \rightarrow ONOOH$$
$$ONOOH \rightarrow \cdot OH + NO_2^\cdot$$
$$\cdot OH + NO_2^\cdot \rightarrow NO_3^- + H^+$$

The production of such powerful oxidizing molecules may well contribute to the cytotoxic, and the neurotoxic effects of $NO\cdot$. $\cdot O_2^-$ and $NO\cdot$ could be released separately from different cell types to form $ONOO^-$ intravascularly or in extracellular compartments. SOD would effectively prevent the reaction between $\cdot O_2^-$ and $NO\cdot$ by scavenging $\cdot O_2^-$ (92). $ONOO^-$ and its proposed decomposition products have recently been defined as potent and potential mediators of vascular injury (91-93), and are suggested to initiate lipid peroxidation and cellular sulfhydryl (SH) oxidation and its depletion (92,94-95).

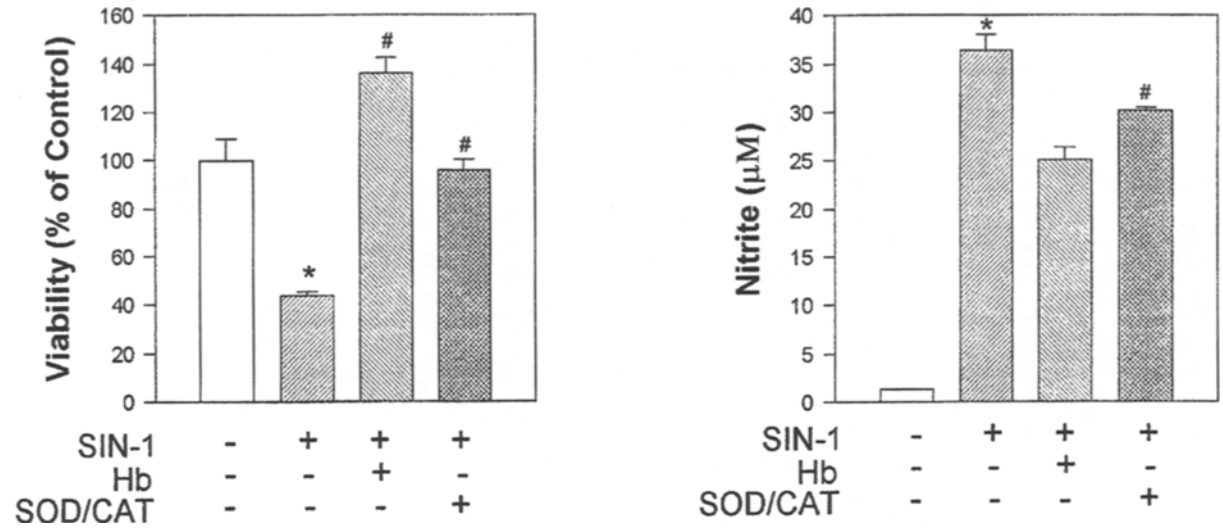

Figure 5. Effects of SIN-1 on endothelial cell viability and nitrite production. Cells were incubated in SFM with SIN-1 and/or Hb or SOD/CAT at 37° for 18-20 hr and the cell viability was measured by MTT assay. The amounts of nitrite were measured by griess reagent. The concentrations of each reagent: SIN-1, 1 mM; Hb, 30μm.; SOD/CAT, 50 units/ml. Each value is the mean ± S.D. of three samples. Statistical significance: *p <0.05 vs control; #p <0.05 vs. SIN-1-treated group. SIN-1, 3-morpholinosynonimine; Hb, hemoglobin; SOD, superoxide dismutase; CAT, catalase; SFM, serum free media.

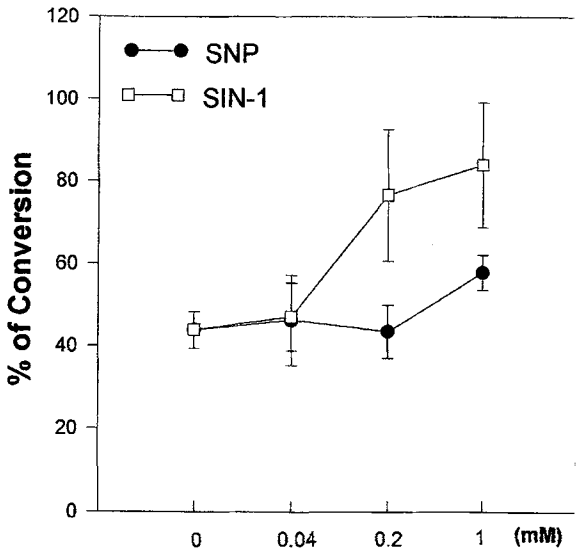

Figure 6. Effects of SNP (NO·) and SIN-1 (·O_2 + NO·) in conversion of XDH to XOD during 1 hr incubation. SNP, sodium nitroprusside; SIN-1, 3-morpholinosynonimine; XOD, xanthine oxidase; XDH, xanthine dehydrogenase; % of conversion, XOD/XDH plus SOD.

NO· is one of the proposed cytotoxic species produced by the immune surveillance system (96,97). Many reports also suggest that during an ischemia reperfusion event, NO· mediates tissue injury (98-105). On the other hand, based on two lines of evidence, NO· has been suggested to function as a protective agent: (i) NO· synthase inhibitors increase tissue damage during in vivo ischemia reperfusion within the cerebral cortex, and (ii), more direct evidence has shown NO· to prevent damage during ischemia reperfusion events in both brain and heart (106-110).

Nitric Oxide Synthase. Nitric oxide synthase (NOS) is classified into three isotypes, identified as types I, II, and III NOS (111-113). Type I NOS, also called neuronal NOS or brain constitutive NOS (bNOS) (114), is an isozyme found in high concentration in some neuronal cells (115-118). The isozyme is activated by calmodulin when intracellular Ca^{2+} is elevated. NO·, produced by type I NOS, is a proposed neurotransmitter in noncholinergic, nonadrenergic transmission (119). Tissue distribution of bNOS mRNA (9.5kb) in rat was reported by Sessa et al. (120). bNOS mRNA is most prominent in brain regions, intestine, stomach, spinal cord, adrenal gland, and aorta. It immigrates with a molecular mass of 150-160 kDa in SDS-PAGE (118).

Type II NOS is referred to as macrophage NOS or inducible NOS (iNOS) (115,117). The enzyme is not present in resting cells, but is induced in a number of cell types by exposure to bacterial lipopolysaccharide in conjunction with cytokines (115). This isozyme of NOS is normally independent of activation by Ca^{2+}. Induction of type II NOS is probably part of the mechanism for mounting a cytotoxic response. NOS II has a denatured molecular mass of 125-135 kDa (112).

Type III NOS, also called endothelial constitutive NOS (ecNOS) (121-124), is the isoform commonly associated with production of endothelial relaxing factor (EDRF) (117,119). Like type I NOS, this isozyme is activated by raised intracellular Ca^{2+} concentration and calmodulin (125) binding to the enzyme. Tissue distribution of eNOS mRNA (4.4kb) was also detected by Sessa et al. (120), who reported that eNOS mRNA is most abundant in aorta, heart, lung, kidney, adrenal

gland, spinal cord, and urogenital tissues. The enzyme shows a denatured molecular mass of 135 kDa (112).

Cras et al. have reported that eNOS and iNOS expression is increased in the lungs of rats subjected to chronic hypoxia (126). Tanaka et al. proposed that L-arginine-NO synthase pathway is activated during reperfusion after focal cerebral ischemia and indicate the involvement of a reaction between NO and $\cdot O_2^-$ during early reperfusion (127).

Cooperative Action of NO· and $\cdot O_2^-$. Cazevieille et al. demonstrate for the first time a clear sequence of events in hypoxic neurotoxicity and emphasize the role of NO· in this toxicity (128). Although the mechanism of NO· action is not fully understood, NO·, which is synthesized in the nervous system under physiological conditions (129), seems to produce deleterious effects in particular circumstances (e.g., ischemia). These toxic effects appear unrelated to physiological NO·-dependent activation of guanylate cyclase (130), but could be caused by the formation of cytotoxic peroxynitrite (131), which reacts with sulfhydryls and membrane lipids (132,133), or could generate hydroxyl radicals (134). This attractive exploration is consistent with Cazevieille's results (128), showing some complementary activity for SOD and NOS inhibitor (NG-nitro-L-arginine), against glutamate toxicity and ischemic neurotoxicity.

We explored the interaction of NO· and $\cdot O_2^-$ on endothelial cytotoxicity by using 3-morpholinosynonimine (SIN-1), which can simultaneously produce NO· and $\cdot O_2^-$ (135). SIN-1 (1 mM) significantly decreased cell viability to 43 ± 1.73% compared to control, with the amounts of nitrite measuring 36.43 ± 1.52 μM. Hemoglobin (Hb, 30 μM) significantly reversed cell viability to 136 ± 6.27%, and the amounts of nitrite decreased to 18.33 ± 12.84 μM. SOD (50 units/ml)/catalase (50 units/ml) also increased cell viability to 95 ± 4.43%. $ONOO^-$ produced from SIN-1 caused endothelial cell injury, but Hb or SOD/CAT abolished the injury, as shown in Figure 5. Here, results suggest that endothelial cytotoxicity is due to the cooperative action of NO· and $\cdot O_2^-$.

To elucidate the possible implication of NO· and $\cdot O_2^-$ in the conversion of XDH/XOD, a NO· generator, sodium nitroprusside (SNP), and SIN-1 were used. As shown in Figure 6, NO· has little effect on the conversion of XDH to XOD, while $\cdot O_2^-$ and NO·-generating, SIN-1, markedly increased the conversion. These results revealed the possibility that oxidizing molecules, such as ·OH from $ONOO^-$, may play a role in regulating the conversion of XDH to XOD, and may lead to ischemic tissue injury.

From the above observations, we may learn that the interaction between $\cdot O_2^-$ and NO· may become significant in tissue injury and cytotoxicity in pathogenic conditions, such as ischemia and inflammation.

Summary of XDH/XOD Status in Aging

In aging, free radicals and other reactive species appear to play an important role in cellular damage and functional degeneration (136). The accumulation of damaged lipids, proteins, and DNA indicate that the oxidative stress of organism is elevated during aging (137). Currently, substantial data show that mitochondria are a major source of reactive oxygen species in the aging cells. However, the participation of XOD has received very little attention as a source of free radicals in the aging process.

One early investigation of XOD in aging is that of Joseph et al. (138), who reported an age-related increase of XOD activity in rat liver. More recently Janssen et al. (139) measured XOD and XDH activity in myocardial tissue of the Wistar rat, finding a slight decrease of XDH activity during aging, and a significant increase of XOD activity at 18 months, compared to rats at 2 months. In this study, XDH and XOD activities were found relatively constant during aging (139).

Our laboratory investigated the effect of age on hepatic XDH/XOD in Fischer 344 rats. The results showed that XOD activity changed little with age, but that XDH activity decreased with increasing age (140). Conversion of XDH to XOD, expressed as a ratio of XOD/XDH, showed only a slight increase during aging. Cytosolic fraction, which contains XOD activity, has many antioxidants such as SOD, catalase, GSH peroxidase, and small molecular antioxidants.

These observations show XOD to play an important role in cellular oxidative status, detoxification of aldehydes, oxidative injury in ischemia-reperfusion, and neutrophil mediation. Furthermore, the synergistic interaction of $\cdot O_2^-$ and NO· may be involved in tissue injury and cytotoxicity in an emergent condition such as ischemia or inflammation.

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