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Free Radical in Ischemia/reperfusion Injury

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Abbreviations

IR	Ischemia reperfusion
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SOD	Superoxide dismutase
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase

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Abstract

The role of ROS in ischemia reperfusion injury was supported by the accumulating evidences. Direct evidence was that oxidant formation has been demonstrated during reperfusion of the ischemic bowel by ESR and chemiluminescence while indirectly evidence was that ROS scavengers including enzymes and small molecules have been shown to protect organs from ischemic reperfusion injury. XDH/XO was demonstrated to be an important source of the oxidants produced after reperfusion though there still exist some contradicts in this theory including the location of XDH/XO, conversion between XDH and XO and involvement of other oxidase. Neutropils were another main source of ROS production and were shown to be the final pathway through which much of the destruction occurs. Some evidences showed that neutrophils adhesion was mediated by ROS. Nitric oxide pathway was also play an important role in ischemic reperfusion injury.

Introduction

The reperfusion of previously ischemic tissue has been shown to be associated with exacerbation of cellular injury as judged by morphologic, physiologic, and biochemical criteria. Reperfusion occasionally potentiates release of intracellular enzymes, influx of Ca_2^+, breakdown of sarcolemmal phospholipids, and disruption of cell membranes, which either alone or in combination result in ultimate cell death [1]. These sequelae of events are known as reperfusion injury, because these changes specifically occur during reperfusion rather than as a result of biochemical changes occurring during ischemia.

The concept of reperfusion injury was first formulated as early as 1935and in 1973 Hearse *et al* [2] demonstrated the release of intracellular enzymes in the ischemic reperfused heart. Hearse and his coworkers were probably the first to term this injury reperfusion injury. After this, the concept of reperfusion injury was spread, and scientists began to realize that any tissue or cell undergoing ischemia may be subjected to reperfusion injury.

There are at least three components of reperfusion injury-microvascular injury, cell necrosis, and hemorrhage. Current evidence leads to at least three major hypotheses concerning the mediators of reperfusion injury [1]: (1) the free radical hypothesis; (2) the calcium overloading hypothesis; and (3) the loss of sarcolemmal phospholipids hypothesis. Of these, the free radical hypothesis has been the most popular because of the presence of both direct and indirect evidence to support it. The presence of hydroxyl radicals in an ischemic reperfused heart has been demonstrated using both electron spin resonance spectroscopy and high-performance liquid chromatography-electrochemical detection technique. Increased generation of lipid peroxidation products such as lipid endoperoxides and hydroperoxides, specifically malonaldehyde, and the formation of

conjugated dienes also support the free radical hypothesis. In addition, many free radical scavengers and antioxidants have been shown to reduce the reperfusion injury.

Reactive oxygen species in reperfusion injury

The observation that reperfusion of ischemic organs leads to tissue dysfunction and/or injury led to the concept that reperfusion may be mediated at least in part by the formation of reactive oxygen species (ROS). Molecular oxygen can accept a total of four electrons to form water; however, it can be reduced in univalent steps to generate three oxidant species: superoxide, hydrogen peroxide, and the hydroxyl radical. The univalent reduction of molecular oxygen results in the formation of superoxide, which is not a highly reactive species. Hydrogen peroxide may be formed by the divalent reduction of oxygen or by the spontaneous dismutaion of superoxide. The latter reaction occurs very rapidly, consequently, the production of $O_2^{\bullet-}$ in vivo is generally accompanied by the production of H_2O_2. The hydroxyl radical ($OH^{\bullet}$), which formed by the interaction of $O_2^{\bullet-}$ and H_2O_2, is highly reactive with a variety of cellular components. The result of oxygen radical formation is damage to an entire array of biomolecules found in tissues, including nucleic acids, membrane lipids, enzymes, and receptors [3]. Membrane-associated polyunsaturated fatty acids (PUFAs) are readily attacked by $HO^{\bullet}$ in a process that results in the peroxidation of lipids. Peroxidation of membrane lipids can alter membrane fluidity and disrupt cell compartmentation, which can result in cell lysis. Thus, ROS-initiated lipid peroxidation and protein damage may contribute to the impaired cellular function and necrosis associated with reperfusion of ischemic tissues.

Evidence for the involvement of ROS in reperfusion ischemia is based on the use of agents that either restricts the production of these cytotoxic oxidants or that act as scavengers after the oxidants are produced. Oxidant formation has been demonstrated during reperfusion of the

ischemic bowel by ESR and chemiluminescence [4, 5]. These studies demonstrate that there is a burst of oxidant formation immediately after reperfusion, which lasts for 2 to 5 min. The enhanced photoemission observed with chemiluminescence after reperfusion is suppressed by treatment with SOD, an enzyme that scavenges $O_2^{\bullet-}$. The concept of reperfusion -induced oxidant production is also supported by the fact that the consumption of reduced glutathione in intestinal mucosa subjected to ischemia- reperfusion is largely prevented by SOD [6].

There is evidence that oxidants mediate both the increased microvascular permeability produced by 1h of ischemic reperfusion and the mucosal lesions produced by 3h of ischemic reperfusion. Both SOD and copper diisopropyl salicylate (Cu-DIPS), a superoxide dismutase mimetic, significantly attenuate reperfusion-induced increase in microvascular permeability. Furthermore, administration of SOD before reperfusion attenuates the necrosis of mucosal villi and crypt epithelium produced by longer period of ischemia [7, 8].

Although the aforementioned studies implicate the superoxide anion in mediating reperfusion -induced intestinal injury, there is evidence that oxidants derived from superoxide play a more important role in the injury process. Catalase, an enzyme that catalyzes the disproportionation of H_2O_2 to H_2O and O_2, has proved to be protective in many models of ischemia-reperfusion. Nonenzymatic scavengers of $OH^{\bullet}$, including DMSO, dimethyl thiourea, and mannitol, also attenuate reperfusion-induced injury [9]. Inasmuch as $OH^{\bullet}$ is formed from O_2^- and H_2O_2 and $OH^{\bullet}$ scavengers provide levels of protection similar to that observed with SOD or catalase, many investigators have suggested that the secondarily derived $OH^{\bullet}$ may be the primary damaging radical. The protective effects of SOD, catalase, and $OH^{\bullet}$ scavengers are consistent with the view that the highly cytotoxic $OH^{\bullet}$ is formed during reperfusion by the Haber-Weiss reaction.

It is generally assumed that the Haber-Weiss reaction occurs at too low a rate to be of physiological significance. However, the reaction can be greatly accelerated by the presence of transition metals, which act as catalysts. Thus, superoxide could release ferrous iron for reaction with hydrogen peroxide to form the $OH^{\bullet}$ during reperfusion. The role of iron in reperfusion-induced $OH^{\bullet}$ production has been assessed by determining whether deferoxamine or apotransferrin provides protection of against the increased microvascular permeability produced by ischemia-reperfusion [10]. Pretreatment with either deferoxamine or apprtransferrin significantly attenuates the increase in microvascular permeability after reperfusion. The observation that iron-loaded deferoxamine or transferring does not protect the intestine against reperfusion injury argues against a nospecific protective effect of these iron-binding compounds. The ability of $OH^{\bullet}$ to initiate lipid peroxidation can result in the formation of lipid derived free radicals such as conjugated dienes, lipid hydroperoxide radicals, and lipid hydroperoxides. Measurement of conjugated dienes and malondialdehyde are frequently used as an index of lipid peroxidation. It was found that malondialdehyde concentrations in both intestinal mjucosa and plasma were increased threefold to fourfold at 5 min after reperfusion. These observation, along with those described earlier, suggest that the $OH^{\bullet}$ mediates perfusion-induced lipid peroxidation in the intestine [11].

ROS have also been shown to destabilize lysosomal membranes and hence to increase the concentration of lysosomal phospholipases in the vicinity of cellular membranes [12]. In line with the free radical triggered lipolysis by phospholipase theory is the recent observation that α-tocopherol, a well known scavenger of oxygen free radical, completely prevents the post-ischemic rise in arachidonic acid in rat hearts [13]. It has been suggested that enhanced phospholipids hydrolysis, giving yield to the release of arachidonic acid and subsequent

production of eicosanoids, may also be the cause of oxygen free radical formation because the conversion of prostaglandin G2 into prostaglandin H2 generates oxygen free radicals [14].

Role of xanthine oxidase

XO normally exists in nonischemic healthy cells predominantly as an NAD^+-dependent dehydrogenase (XDH). XDH is generally recognized as the terminal enzyme of purine catabolism in man, catalyzing the hydroxylation of hypoxanthine to xanthine and of xanthine to urate (Reaction 1 and 2). This form of the enzyme uses NAD^+ instead of O_2 as the electron acceptor during oxidation of purines and does not produce $O_2^{\bullet-}$ or H_2O_2. XOR is the rate-limiting enzyme in nucleic acid degradation through which all purines are channeled for terminal oxidation.

$$\text{Hypoxanthine} + NAD^+ + H_2O \rightarrow \text{xanthine} + NADH + H^+ \quad (1)$$

$$\text{Xanthine} \rightarrow \text{Uric acid} + 2e^- \quad (2)$$

In 1981, Granger and colleagues focused attention on XO and it was first postulated that xanthine oxidase (XO)-derived oxidants play an integral role in the microvascular injury associated with reperfusion of ischemic intestine [7]. Their hypothetical mechanism, outlined in figure 1, can be briefly summarized as follows. In the course of ischemia, transmembrane ion gradients are dissipated, allowing elevated cytosolic concentrations of calcium. This, in turn, activates a protease that irreversibly converts (xanthine dehydrogenase) XDH, predominant in vivo, into XO. Concurrently, cellular ATP is catabolized to hypoxanthine, which accumulates. On reperfusion, readmitted oxygen, hypoxanthine, and XO combine to generate superoxide and hydrogen peroxide (Reaction 3 and 4).

$$\text{Hypoxanthine} + 2O_2 + H_2O \rightarrow \text{xanthine} + O_2^{\bullet-} + H_2O_2 \quad (3)$$

$$\text{Xanthine} + 2O_2 + H_2O \rightarrow \text{uric acid} + O_2^{\bullet-} + H_2O_2 \quad (4)$$

These reactive oxygen species can interact to yield a range of cytotoxic agents, including hydroxyl radicals [15].

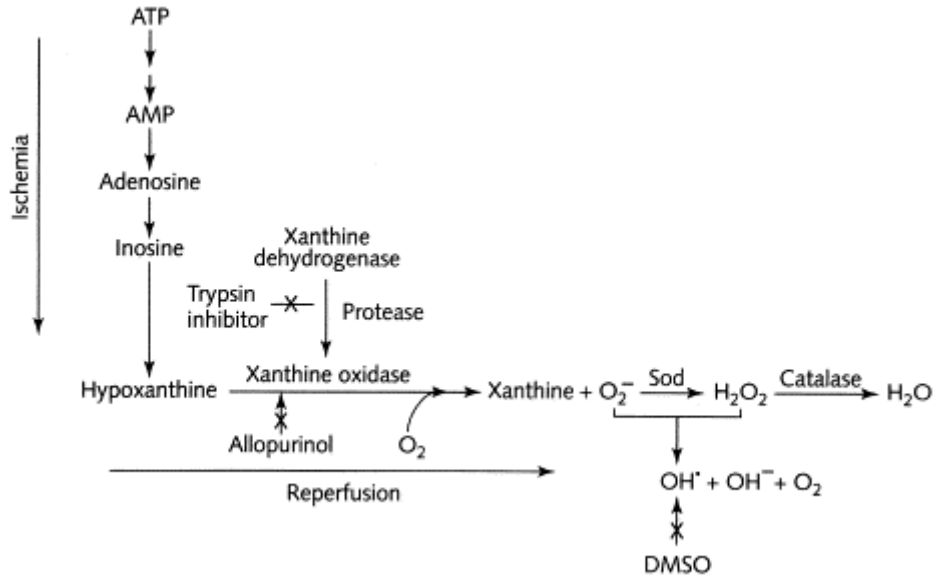

Figure 1 Mechanism of generation of ROS in IR as proposed by Granger et al [15].

Parks et al have studied the conversion mechanism of XDH to the oxidant producing XO during tissue ischemia [16]. They found that Conversion of XDH to XO can occur by two mechanisms: reversible conversion by oxidation or irreversible conversion via limited proteolysis. The XDH-to-XO conversion that occurs during ischemia appears to proceed at different rates in various tissues; however, the amount of XDH-to-XO conversion is proportional to the duration of ischemia.

Since its inception, the XO hypothesis has undergone considerable refinement and it has been tested in virtually every organ system. In humans, liver and intestine have the highest xanthine oxidase (XDH plus XO) activity. Although these levels are relatively low compared with values reported for other mammals, the potential cytotoxicity of this enzyme activity is exemplified by the observation that freshly isolated or cultured cells are injured when expose to xanthine oxidase

levels as low as 2mU/ml [17]. Many studies, particularly the earlier ones, confirmed the proposed role of XO in IR damage in intestine, liver, and kidney [18, 19].

The XO inhibitors allopurinol, oxypurinol and pterin aldehyde have been widely used to assess the contribution of XO. All of the inhibitors dramatically attenuate both the epithelial cell necrosis and the increase microvascular permeability observed after reperfusion of the ischemic bowel, suggesting that XO is an important source of the oxidants produced after reperfusion [20]. However, at relatively high concentrations (>500 μM), allopurinol and oxypurinol have been shown to act as powerful scavengers of hydroxyl radicals in vitro and the possibility that the beneficial effects of these inhibitors could result from such scavenging has been examined [21]. Although the ability of allopurinol to attenuate reperfusion injury is generally attributed to the decreased production of XO-derived oxidants, preservation of the nucleotide pool is often raised as an alternate explanation. Allopurinol does appear to attenuate the reduction in cellular ATP produced by intestinal ischemia; however, maintenance of ATP levels by administration of inosine does not protect the cat small intestine against reperfusion injury. Additionally, the fact that administration of allopurinol at the time of reperfusion is as effective as pretreatment in attenuation reperfusion injury argues against a role for purine salvage [22].

Additional evidence in support of this theory is provided by studies in which tissue XO was depleted by administration of a tungsten-supplemented, molybdenum-deficient diet [20]. Tungsten replaces molybdenum in the active site of XO, rendering the enzyme inactive. Feeding cats a tungsten-supplemented diet results in a 75% reduction in mucosal XO activity and a corresponding attenuation of the reperfusion induced increase in intestinal microvascular permeability [20]. Although this approach also demonstrates a role for XO, its interpretation is limited in the other molybdenum-containing enzymes such as aldehyde oxidase are also

inactivated by this diet. A role for XO in reperfusion injury is also supported by the observation that local intra-arterial infusion of hypoxanthine and XO results in increase in intestinal vascular permeability comparable to that produced by ischemia and subsequent reperfusion [23].

Perhaps most controversial has been the extent and time scale of XDH to XO conversion in ischemic tissues, particularly liver. While this conversion occurs, it is too slow to play a major role in IR-induced tissue damage [24, 25]]. In fact, it can be argued that XDH to XO conversion is not essential to involvement of XOR in IR. Upregulation of overall XOR activity as a consequence of ischemia and/or of substrate level changes could equally well lead to increases in ROS [26].

The role of XOR in IR injury of the heart has been especially contentious, largely because of the very low activities of XOR detected in rabbit, pig, and, particularly, human hearts [27]. The brain also has been much discussed in this context, with evidence both for and against a major involvement of XOR in IR injury [28]. It is worth bearing in mind that, because XOR is most probably localized to certain cell types (e.g., those of the vasculature), it is levels and changes of the enzyme within these cells that have relevance to IR. Such information is not, in general, available from studies on whole tissue. A further point is that circulating XOR, derived from high-activity tissues, such as liver, could be concentrated in the vasculature of lower activity tissues, such as heart, and there effect injury.

Role of neutrophils in reperfusion injury

The importance of the neutrophil in ischemia/reperfusion injury has been recognized for many years. Neutrophils were once considered the “trigger” of ischemia/reperfusion injury. More

recent work has characterized the neutrophil as the "bullet" or final pathway through which much of the destruction occurs [29]. Neutrophils are known to produce O_2^- and H_2O_2 and to secrete myeloperoxidase, an enzyme that catalyses the formation of hypochlorous acid from H_2O_2 and chloride ions [30]. Activated neutrophils also produce potent proteases capable of degrading virtually all components of the endothelial basement membrane as well as junctional proteins that maintain endothelial barrier function [31]. Leukocytes exposed to ischaemic tissue may re-enter the systemic circulation in an activated state upon reperfusion. These activated neutrophils have been implicated as mediators of I/R-induced remote organ injury [31].

1.Neutrophils as a source of ROS

Neutrophils contain NADPH oxidase that reduces molecular oxygen to the superoxide anion. Lactoferrin, stored in and released from specific granules of neutrophils, plays a role in the process of oxygen metabolism as an iron donor. Activated neutrophils also secrete the enzyme myeloperoxidase, which catalyzes the formation of hypochlorous acid from hydrogen peroxide and chloride ions:

$$H_2O_2 + Cl + H^+ \rightarrow HOCl + H_2O \qquad (5)$$

The hydrogen peroxide needed to form HOCl is derived from the spontaneous dismutation of NADPH-derived superoxide. Several methods have been used to define the role of neurophils in reperfusion tissue injury, including monitoring the appearance of radiolableled neutrophils or neutrophil-specific enzymes such as MPO. It has been demonstrated by measurements of mucosal MPO activity in cat intestine hat there are approximately 10 million neutrophils per gram of tissue [32]. When these cells are maximally activated, they can generate a superoxide flux of 35nmol/minute per gram of tissue, which is extremely cytotoxic.

HOCl is a potent oxidizing and chlorinating agent that is approximately 100 times more reactive than H_2O_2. it reacts rapidly with primary amines(RNH_2) to produce N-chloro derivatives (RNHCl), which have the oxidizing equivalence of H_2O_2 and HOCl.

$$HOCl + RNH_2 \rightarrow H_2O + RNHCl \qquad (6)$$

The lipophilic N-chlormines are likely causes of the cellular injury associated with HOCl production. The cytotoxicity associated with HOCl and N-chloromines may be mediated via the oxidation of sulfhydryls, inactivation of heme protein and cytochrome, and degradation of amino acids and proteins [33]. Although RNHCl do not contain an atom of oxygen, it can be seen that RNHCl are ultimately derived from superoxide anion. Some investigators have proposed that HOCl may mediated cytotoxicity indirectly by its ability to inactivate α1-protease inhibitor while activating a neutrophil-associated collagenase and gelatinase [34]. Because activated neutrophils can release a variety of enzymes including elastase and collagenase, it is possible that neutrophil-derived oxidants enhance the destructive potential of neutronphils in the microcirculation by activating neutrophil-associated proteases.

2.Neutrophils infiltration

Estimate of neutrophil infiltration into the postischemic intestinal mucosa have been derive from biochemical determinations of tissue MPO activity. A fivefold to sevenfold increase in MPO activity was observed in mucosal enzyme activity during the ischemic period, whereas reperfusion produced an 18-fold enhancement of activity. Treatment with either SOD or allopurinol significantly attenuated the mucosal MPO activity observed after reperfusion [35]. The neutrophil accumulation initiated by reperfusion is also significantly attenuated by treatment with either catalase, dimethylthiourea(OH scavenger), or deferoxmine[36]. These observations suggest that ROS play a role in recruitment of neutrophils into postischemic intestinal mucosa.

Intravital microscopic studies of tissues exposed to ischemia and reperfusion have revealed an acute inflammatory response that is characterized by enhanced protein efflux and an increased adherence and emigration of leukocytes in postcapillary venules [37]. A role for leukocytes as mediators of the microvascular dysfunction elicited by ischemia followed by reperfusion is supported by whole organ studies that demonstrate: (1) an accumulation of neutrophils in postishcemic tissues, (2) attenuation of reperfusion-induced vascular injury in animals rendered neutropenic with neutrophil antiserum, and (3) a reduction in reperfusion-induced vascular leakage by monoclonal antibodies that prevents leukocyte adhesion [35]. The latter observation has led to the recognition that leukocyte-endothelial cell adhesion may be rate-limiting step in the pathogenesis of reperfusion-induced tissue injury.

Additional support for the view that XO-derived oxidants mediate reperfusion-induced neutrophil infiltration is provided by intrvital microscopic studies of leukocyte-endothelial cells adhesion in postischemic mesenteric venules that demonstrate that allopurinol and SOD attenuated the increases in leukocyte adherence and emigration elicited by ischemia and reperfusion. Moreover, it has been shown that catalase, SOD and oxypurinol significantly attenuate leukocyte adherence, while neither inactivated catalase nor desferrioxamine altered the reperfusion-induced leukocyte adhesion [38]. These results are consistent with the view that XO-derived oxidants contribute to the leukocyte-endothelial cell adhesive interactions associated with reperfusion of ischemic tissues.

Nitric oxide and reperfusion injury

Much of the data derived from studies of IR-induced microvascular dysfunction are consistent with a role for both superoxide and NO as participants in the altered endothelium-dependent responses [39]. Nitric oxide (NO) is a biologically active substance produced by endothelial cells,

and it is inactivated by superoxide [40]. NO has been shown to inhibit neutrophil aggregation i*n vitro*, an effect that is potentiated by the addition of SOD. One would predict that decreased NO production or increased NO inactivation would promote leukocyte adherence. Because superoxide inactivated NO, conditions associated with an increased superoxide production (*i.e.* ischemia/reperfusion) would be expected to enhance leukocyte adherence, and SOD should be anti-adhesive. It has been demonstrated that SOD attenuates leukocyte adhesion in mesenteric venules and NO affords protection against intestinal reperfusion injury [40]. These observations are consistent with the hypothesis that the beneficial actions of SOD may in part be attributed to its ability to prevent NO inactivation.

The available data also suggest that IR induces the impaired endothelium-dependent vasodilation in arterioles as well as the acute inflammatory response in venules by altering the balance between NO and superoxide in endothelial cells. The prevailing view is that under normal conditions, the flux of NO greatly exceeds the rate of superoxide production. This allows for NO (1) to effectively scavenge the low intracellular levels of superoxide; (2) to reduce arteriolar tone via guanylate cyclase activation in smooth muscle; (3) to prevent platelet aggregation and thrombus formation; and (4) to minimize the adhesive interactions between leukocytes and the endothelial cell surface. However, within minutes after reperfusion of ischaemic tissues, the balance between NO and superoxide is tipped in favour of superoxide. This imbalance results from a profound increase in the production of superoxide by endothelial cells (and adherent leukocytes) and a corresponding decline in the synthesis of NO from endothelial NO synthase. The relatively low levels of NO that are produced by endothelial cells react with the abundant supply of superoxide, leaving little or no bioactive NO to oppose blood cell-endothelial cell interactions and to maintain optimal tissue blood flow. In addition, endothelium-dependent

vasodilation is compromised because NO is not available to serve as a second messenger when certain endogenous vasodilators (acetylcholine) interact with their endothelial receptors.

The accumulation of superoxide that occurs in the absence of NO after IR allows for an enhanced generation of hydrogen peroxide (superoxide reacts with NO much faster than the rate of superoxide dismutation). The two reactive oxygen metabolites (O_2^- and H_2O_2) can rapidly initiate or exacerbate the inflammatory state in venules (1) by eliciting the production of platelet-activating factor, via phospholipase activation; (2) by promoting the activation and deposition of complement on the endothelial cell surface; and (3) by mobilizing the stored pool of P-selectin to the endothelial cell surface, where it mediates leukocyte rolling. Reactive oxygen metabolites also help to sustain the leukocyte-endothelial cell adhesion that occurs several hours after reperfusion by activating genes that encode adhesion molecules such as E-selectin (sustains leukocyte rolling) and ICAM-1 (sustains firm adhesion and emigration of leukocytes). The oxidant-dependent activation of genes for specific endothelial cell adhesion molecules genes is mediated by nuclear transcription factors such as NFκB and AP-1, which in turn are activated by an oxidant stress. The transcription-dependent synthesis of the endothelial cell adhesion molecules ensures that the inflammatory responses elicited by IR can occur for several hours after the initiation of reperfusion and the NO-superoxide imbalance.

Some limited evidence exists that the up-regulation of eNOS protects against IR injury under pathological or physiological conditions. In experimental models of cardiac IR injury, up-regulation of eNOS has been found and in transplant studies, eNOS up-regulation by gene transfer was protective against IR injury [41].

Hypotheis and experimental design

From the above description and evidences, we can draw the conclusion that ROS plays an important role in ischemic reperfusion injury. The hypothetic pathway of ROS in ischemic reperfusion is as following:

(1) During ischemic, XDH is converted to XO

(2) After reperfusion, ROS is produced by XO

(3) ROS decreases NO concentration

(4) Increased ROS and decreased NO induce neutrophils adhesion

(5) Activated neutrophils produce more ROS

(6) ROS production result in microvascular dysfunction and tissue injury.

In this hypothesis, ROS production could be divided into two stages. At the first stage, activated XO generates a small amount of ROS, which is not enough to cause comprehensive ischemic reperfusion injury. However the ROS produced in this stage is very important in neutrophils adhesion and infiltration. The latter stage ROS is generated by neutrophils and cause microvascular dysfunction and tissue injury.

However, some aspects of this hypothesis still need to be testified. I want to further study three of them

1. Ischemic reperfusion injury in Knock-out animal

Animal model is very important in ischemic reperfusion study because this is a complicated pathological process in vivo. In order to demonstrate the role of XO, SOD and eNOS in ischemic reperfusion injury, I want to construct XDH, SOD and eNOS knock-out animals respectively. Using normal animals as control, I hope I could find the difference in ischemia reperfusion

process in knock-out animals. The difficulty of these experiment lies in that the physiology of knock-out animal may differ from that of normal one. After standardization, in XDH knock out animals, the decreased ischemia reperfusion injury should be observed while in SOD or eNOS knock-out animals, the injury exaggerate.

2. Time course of ROS production

In this experiment I wan to know the real time ROS changes during the ischemic reperfusion. The key point is to design an *in vivo* real time ROS probe and monitor system. Whole body EPR machine may be used to monitor the overall ROS changes in the region of ischemic reperfusion. In addition, superoxide, hydrogen peroxide, NO and peroxynitrite need be monitored respectively so we will know the exact situation of ROS and RNS production in the ischemia reperfusion region.

3. Applications of small molecular ROS scavenger in ischemia reperfusion

Though some evidence showed that enzymes such as SOD, catalase and eNOS through gene transfer could protect organs from ischemia reperfusion injury, this method is irrealizable in application. So small molecular ROS scavengers such as glutathione, NAC are promising in treatment of ischemia reperfusion injury. In this experiment, I want to further demonstrate the protection role of small molecular ROS scavengers including which one is most effective, and most important, when is the optimum time for administration.

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