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Association of Free Radicals and the Tissue Renin-Angiotensin System: Prospective Effects of *Rhodiola*, a Genus of Chinese Herb, on Hypoxia-Induced Pancreatic Injury

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Summary

The renin-angiotensin system has long been recognized as crucial factor in the regulation of the systemic blood pressure and renal electrolyte homeostasis. Numerous studies have demonstrated the presence of a local renin-angiotensin system in a variety of organs. A recent study of the pancreatic renin-angiotensin system showed that chronic hypoxia significantly increased the mRNA expression for angiotensinogen II receptor subtypes AT_{1b} and AT_2. The activation of the renin-angiotensin system may play an important role in cellular pathophysiological processes. Angiotensin II enhances the formation of reactive oxygen species via the activation of xanthine oxidase or NAD(P)H oxidase. The reactive oxygen species can cause oxidative damage in the pancreas and other tissues either directly or indirectly via the formation of other radicals such as reactive nitrogen species. *Rhodiola* therapy may protect hypoxia-induced pancreatic injury in two ways. It prevents hypoxia-induced biological changes by increasing intracellular oxygen diffusion and efficiency of oxygen utilization. Alternatively, it reduces hypoxia-induced oxidative damage by its antioxidant activities. Additional experimental data are required to fully elucidate the mode of action of this herbal drug.

Introduction

The renin-angiotensin system (RAS) has long been recognized as crucial in the regulation of the systemic blood pressure and renal electrolyte homeostasis. In 1898, Tigersted and Bergmann discovered renin in rabbit kidney extract which, when injected into rabbits, increased blood pressure [1]. Over the last decade, several components of the RAS have been discovered in a variety of tissues [2]. Under conditions of salt, volume loss, or sympathetic activation, the protease renin is released by the kidney and cleaves liver-derived angiotensinogen to form angiotensin I in the circulating blood. Consequently, angiotensin I is converted into angiotensin II mainly by angiotensin-converting enzyme (ACE). Angiotensin II is the main effector octapeptide which increases blood pressure directly via vascular receptors and indirectly through facilitation of the vasoconstrictor action of the sympathetic nervous system [2].

At least two major angiotensin II receptors, namely AT_1 (AT_{1a} and AT_{1b} subtypes) and AT_2, have been described. The AT_1 subtype mediates the physiological functions of angiotensin II, including hypertrophy, vasoconstriction, and the release of catecholamines from sympathetic nerve endings [3]. The AT_2 subtype is coupled to a phosphotyrosine phosphatase, and is the

dominant receptor expressed in the uterus, adrenal medulla and heart [3, 4, 5].
Based on the discrepancies between RAS blocker-induced changes in the circulating levels of the RAS components and the ability of these receptor blockers to lower the blood pressure, the local RAS was proposed in addition to the circulating RAS. The presence of renin substrate, renin-like enzymatic activity and ACE has been demonstrated in various tissues by means of immunohistochemical and biochemical techniques [3]. Subsequent studies have demonstrated the presence of a local RAS in a variety of organs such as the heart, kidney, adrenal gland, brain, pancreas, and in blood vessels [6, 7, 8, 9, 10, 11].
The expression of major RAS genes can be activated by a number of factors such as hormones and different kinds of stress [12]. Hypoxia is one kind of stress that has been shown to upregulate local RAS components. Recently, a detailed study of pancreatic RAS showed that chronic hypoxia significantly increased the mRNA expression for angiotensinogen, AT_{1b} and AT_2 receptors [13].
It has been reported that the production of free radicals can be increased by hypoxia in the liver [14]. However, it is still unclear whether such a phenomenon can be observed in the pancreas. The possibility that hypoxia may induces oxidative damage in the pancreas and its relationship to the activation of pancreatic RAS will be discussed. In addition, the prospective effects of *Rhodiola*, a genus of Chinese herb, on hypoxia-induced pancreatic injury will be evaluated.

Antioxidant Network

To counteract the potential hazard caused by free radicals, an intricate antioxidant system consisting of both enzymes and small molecules has developed during the course of evolution. Several biological mechanisms against free radicals toxicity have been reported, including highly specific electron transfer pathways, radical scavengers, detoxification enzymes and repair systems [15, 16]. For the non-enzymatic antioxidant system, many free radical scavengers, such as glutathione (GSH), uric acid, beta-carotenes (vitamin A), ascorbic acid (vitamin C) and tocopherols (vitamin E), have been found in cellular systems. These scavengers react directly with free radicals to produce more stable and less toxic products [17].
The enzymatic antioxidant system includes a class of detoxification enzymes. Superoxide dismutases catalyze the dismutation of superoxide anion to hydrogen peroxide. Catalase reduces hydrogen peroxide to water and oxygen and thus prevents the formation of the more reactive hydroxyl radical [18, 19]. Selenium-glutathione peroxidase catalyzes the decomposition of hydrogen peroxide and organic hydroperoxides with the consumption of GSH. Glutathione S-transferases are a family of enzymes that catalyze the reduction of lipid hydroperoxides and the conjugation of electrophilic xenobiotics with GSH to produce less toxic and more water-soluble derivatives for excretion. These reactions result in the accumulation of glutathione disulfide (GSSG) which can crosslink cellular macromolecules and thus impair enzyme-mediated reactions. Nevertheless, GSSG can be reduced intracellularly to GSH by glutathione reductase at the expense of NADPH generated from the pentose phosphate pathway [16, 20].
GSH plays a central role in these reactions. GSH can react directly with hydroxyl radicals and reactive nitrogen species formed from the reaction of superoxide radical and nitric oxide (NO). It regenerates vitamin C and vitamin E from their oxidized forms [21]. It also acts as a substrate for the detoxification of hydrogen peroxide and lipid peroxide catalyzed by glutathione peroxidase and glutathione-S-transferase [16, 20]. Apart from the regeneration from GSSG, the consumed GSH can be replenished by intracellular synthesis (Reactions 1 and 2) from its constituent amino acids which are catalyzed by gamma-

glutamylcysteine synthetase and glutathione synthetase, respectively [22].

Reaction 1:

L-Glutamate + L-Cysteine + ATP ⟶ L-gamma-Glutamyl-L-cysteine + ADP + Pi

Reaction 2:

L-gamma-Glutamyl-L-cysteine + Glycine + ATP ⟶ Glutathione + ADP + Pi

Effects of Hypoxia on Oxidants and Antioxidants

It is reasonable to expect that reactive oxygen species (ROS) production will be reduced under low oxygen tension. However, experimental data have shown that various disturbances resulting from hypoxic stress led to lipid peroxidation and cellular damage [23]. Under hypoxic conditions, cellular components become more reduced and may produce ROS by donating electrons either directly or indirectly to oxygen molecules, also known as reductive stress [24]. In addition, chronic hypoxia may impair endogenous antioxidant defense and render the cells more susceptible to subsequent oxidative injury induced by xenobiotics [25].

The rate of GSH synthesis and the level of GSH have been reported to be affected by the various extent of hypoxia. The level of hepatic GSH was decreased by 25% in rats exposed to 7% oxygen [14] and the rate of GSH synthesis was reduced when rats were exposed to 10.5% oxygen for 10 days [26]. Since the synthesis of each GSH molecule requires two molecules of ATP (Reactions 1 and 2), the decrease of GSH synthesis may be due to the limited supply of ATP during hypoxic conditions. For rats exposed to 10.5% oxygen, the cellular GSH level was unchanged although the maximal GSH synthesis was decreased [26]. It may suggest that the level of GSH is highly dependent on the extent of hypoxia. Under mild hypoxic conditions, the synthesized GSH may be able to maintain its regulated level of GSH. However, when GSH is required for the detoxification processes, the GSH level will be decreased drastically. It is evidenced by the observation that cells were more susceptible to oxidative injury under hypoxic condition [26]. The phenomenon may result from a combination of deteriorated factors including the decrease of GSH synthesis, the accumulation of GSSG, and the impaired GSH-dependent detoxification reactions in the hypoxic condition where the supplies of ATP and NADPH are inadequate.

It has been reported that the activity of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase decreases during hypoxia [27]. However, other studies showed that hepatic superoxide dismutase activity was unchanged in rats exposed to 10.5% oxygen for 10 days [26] and increased by 65% in rats during moderate intermittent hypoxia [14]. The discrepancy in these results may suggest that the activity of antioxidant enzymes is highly dependent on the experimental conditions and the duration of hypoxia. Acute hypoxia may cause oxidative damage while mild and chronic hypoxia may induce an adaptive response.

Hypoxia-induced oxidative damage in vascular tissues has been reported in many studies. Hypoxia increased leukocyte sequestration in isolated hearts [28] and adherence of leukocytes to cultured endothelial cells and incubated umbilical vein cells [29, 30]. The increased endothelial adhesive reaction of the leukocytes during hypoxia has been suggested to be mediated by the decrease of the NO level. Experimental results have shown that the production of NO was decreased in endothelial cells during hypoxic conditions *in vitro* and in isolated organs [31, 32]. NO is released from the endothelial cells and plays an important role in the regulation of vascular functions. It acts as an anti-inflammatory mediator which prevents the adhesion of leukocytes to vascular endothelial cells [33, 34, 35]. The decrease of the NO level during hypoxia may be mediated by the reduction of NO synthesis and the increase of NO degradation. Since the oxygen molecule is a substrate in the NO synthesis, it is no doubt that the synthesis is reduced during

hypoxia. Besides, superoxide anion radicals produced during hypoxia can also cause a decrease of the NO level in vascular cells [36]. The superoxide radical reacts with the NO to form peroxynitrite (Reaction 3). Peroxynitrite can isomerize to nitrate (Reaction 4), which is relatively inert to cellular molecules [37]. However, peroxynitrite itself can form a strong oxidant that causes cellular damage by reaction with other biomolecules [38]. Peroxynitrite also reacts with the remaining NO to form nitrogen dioxide (Reaction 5). Nitrogen dioxide reacts with NO to form the nitrosating agent N_2O_3 (Reaction 6) [39]. These reactions further deplete the level of NO and thus enhance the adhesion of leukocytes to the vascular endothelial cells.

Reaction 3:

$$NO + O_2^- \longrightarrow ONOO^-$$

Reaction 4:

$$ONOO^- + H^+ \rightleftharpoons ONOOH \longrightarrow HNO_3 \rightleftharpoons H^+ + NO_3^-$$

Reaction 5:

$$ONOO^- + NO \longrightarrow NO_2 + NO_2^-$$

Reaction 6:

$$NO_2 + NO \rightleftharpoons N_2O_3$$

The involvement of free radicals in hypoxia-induced leukocyte-endothelial adhesive interactions was supported by the observation that an intravenous infusion of antioxidants prevented the formation of ROS and the adhesion of leukocytes in rat mesenteric venules during systemic hypoxia [40].

The Tissue Renin-Angiotensin System as an Initiator of Free Radicals

The activation of pancreatic RAS by chronic hypoxia may be a novel mechanism for the initiation of ROS during hypoxic conditions. In particular, angiotensin II may play a central role in initiating free radical production in local tissue via different mechanisms, including the activation of xanthine oxidase (XO) and NAD(P)H oxidase.

Like other vasoconstrictors, a transient increase of local angiotensin II may theoretically lead to classic hypoxia-reoxygenation injury that involves XO-mediated free radicals production [41, 42]. Xanthine oxidoreductase exists in two forms, xanthine dehydrogenase (XDH) and XO. The enzymes catalyze two consecutive reactions, the conversions of hypoxanthine to xanthine and xanthine to uric acid. XDH transfers electrons to the oxidized form of nicotinamide adenine dinucleotide (NAD) while XO transfers electrons to molecular oxygen [43, 44]. Both enzymes generate hydrogen peroxide and superoxide radicals when xanthine and oxygen molecules are used as substrates. However, the production of superoxide radicals by XDH is almost completely inhibited by its electron acceptor, NAD. Therefore, the superoxide radical formed in such a way does not cause significant damage in cells that contain sufficient amounts of NAD [44, 45]. But, the superoxide radical produced from XO will initiate oxidative damage. In normal cells, XDH is the predominant form of xanthine oxidoreductase. However, XDH can be converted to XO either by proteolytic cleavage or the oxidation of sulfhydryl residues which occurs rapidly during [46]. In addition, the depletion of ATP and the accumulation of hypoxanthine by hypoxia enhance the generation of ROS during reoxygenation [42].

Apart from the XO pathway, NAD(P)H oxidase has been suggested as an alternative mechanism of free radical formation initiated by angiotensin II [24]. The membrane-bound-NAD(P)H oxidase complex is a FAD-containing flavoprotein which transfers electrons from NAD(P)H to molecular oxygen. The role of angiotensin II in the formation of superoxide radicals by NAD(P)H oxidase has been demonstrated in endothelial cells, vascular smooth muscle cells and mesangial cells [47, 48, 49]. Although the mechanism for the formation of the superoxide radical by

angiotensin II is not fully understood, it is believed that specific binding of angiotensin II to the AT_1 receptor subtype is a possible initiating process [50]. The binding of angiotensin II to the AT_1 receptor stimulates G-protein-coupled phospholipase C and hydrolyzes membrane phophatidylinositol-4,5-bisphosphate to 1,4,5-inositoltriphosphate and diacylglycerol [51]. The water soluble 1,4,5-inositoltriphosphate stimulates the release of the calcium ion sequestered in the endoplasmic reticulum which, in turn, activates protein kinase C and mitogen-activated protein kinase [52, 53]. Both calcium ions and protein kinase C have been demonstrated to activate NAD(P)H oxidase and elevate superoxide formation [48, 54, 55]. Experimental results from studies of animal and human blood vessels have supported the role of the AT_1 receptor in superoxide production via the activation of membrane-bound NAD(P)H oxidase [48, 56, 57]. The superoxide formed in the vascular cells was blocked by losartan (AT_1 receptor antagonist) and inhibited by diphenyleneiodonium (inhibitor of NAD(P)H oxidase), respectively [56].

The superoxide produced in this way has been suggested to play an important role in the pathophysiological activity of angiotensin II. The superoxide induced by angiotensin II has been shown to alter vasomotor tone [16], induce hypertrophy in renal tubular cells [58] and vascular smooth muscle cells [59], reduce NO-mediated vasorelaxation [60], stimulate atherosclerosis [57], and enhance endothelial cell apoptosis [61, 62]. The apoptotic effect of angiotensin II has been suggested to be mediated by both AT_1 and AT_2 receptors [61]. In contrast to the signal transduction cascade of the AT_1 receptor, the signaling induced by the specific binding of angiotensin II to the AT_2 receptor has not been well established. Apart from the induction of apoptosis, the function of the AT_2 receptor has been implicated in the inhibition of cell proliferation [63, 64, 65], cell differentiation [66, 67] and neuronal regeneration [68]. In addition, the AT_2 receptor has been shown to stimulate the expression of adhesion molecules and increase the adhesion of leukocytes to vascular endothelial cells as is done by the superoxide radicals [53].

ACE catalyzes the conversion of angiotensin I to angiotensin II and the degradation of bradykinin [69]. When angiotensin I was added to cultured human endothelial cells, superoxide formation was reduced by the ACE inhibitor and angiotensin receptor antagonists [24]. The effect of the ACE inhibitor was reversed competitively by the addition of angiotensin II further supporting the role of angiotensin II in stimulation of superoxide formation in local tissues [24].

Since chronic hypoxia significantly activates pancreatic RAS including the increased level of angiotensinogen and the upregulation of the AT_{1b} and the AT_2 receptors, it is possible that chronic hypoxia will increase superoxide radical formation in the pancreas and such radical production may be mediated by the stimulating effect of angiotensin II on the activity of XO and NAD(P)H oxidase.

A Simple Principle of Traditional Chinese Medicine

The human body is considered as a whole in traditional Chinese medicine. The objective of Chinese medicine is to maintain a dynamic balance between the two opposing forces, the Yin and the Yang [70]. Health and disease are considered as a harmony and imbalance of these forces, respectively. Superimposed upon the Yin/Yang theory is the concept of Five Elements. The Five Elements are Metal, Wood, Water, Fire, and Earth that can respectively represent the five “solid” organs (Zang), lung, spleen, kidney, heart, and liver in traditional Chinese medicinal theories. The relationship among the Elements is governed by the law of nature such as water can put out fire. For example, for the symptom of high blood pressure, the therapeutic approach is to treat the kidney (Water) in order to control the heart (Fire). It is somehow consistent with the Western medicine as diuretics acts on kidney

rather than heart to increase urination for the purpose of lowering the blood pressure.

The principle of treatment in traditional Chinese medicine is to harmonize the equilibrium of Yin and Yang by regulating body constituents (Qi, Blood, Essence, and Fluids) among the five-organ network. Weak organs are tonified, excess is dispersed, and congested channels are cleared. In the concept of five "solid" organs, the pancreas is associated with the spleen which has the responsibility of "logistics" (digestive function). A disease arising from the weakness of the pancreas (Earth) can be prevented by strengthening the heart (Fire) or dispersing the kidney (Water), in addition to tonifying the organ itself, because Fire engenders Earth and Water restrains Earth in nature.

Effects of *Rhodiola* on Hypoxia-Induced Pancreatic Injury

Rhodiola is a genus of Chinese herb which originates from alpine plants. Over 70 species of *Rhodiolae* have been reported to exist in China [71]. *Rhodiola* has been used as a tonic, hemostatic, and for contusions in traditional Chinese medicine for thousands of years. In recent years, its adaptogenic properties [72, 73], anti-hypoxia [74] and anti-fatigue effects [75] and enhancement in learning and memory [76, 77] have been reported.

In traditional Tibetan medicine, *Rhodiola* is commonly used for promoting blood circulation and relieving cough [78]. A recent study has shown that oral administration of *Rhodiola* rosea extract at a dose of 3.5 mg/kg in rats prevented ischemia-reperfusion injury in isolated hearts [79]. This treatment also prevented the decrease of the contraction amplitude and the reduction of coronary blood flow during the postischemia period. The protective effect of *Rhodiola* has been suggested to be associated with the increase of endogenous opioid peptides [79]. A study involving electron microscopic observation showed that oral administration of *Rhodiola* kirilowii prevented high altitude hypoxia-mediated damage in rat viscera by improving blood circulation [80]. A similar study showed that the anti-hypoxic effect of *Rhodiola* semenovii was associated with a transient lowering of the arterial pressure, a decrease of the heart rate and a lengthening of the cardiac contraction phase [74]. The anti-hypoxia effect of *Rhodiola* has also been attributed to the increase of the oxygen supply and the decrease of oxygen consumption in the respiring cells and the improved efficiency of mitochondrial respiration in skeletal muscle [81].

The *in vitro* and *in vivo* antioxidant activities of *Rhodiola* have been reported in many studies. A recent study of 19 compounds isolated from *Rhodiola* sacra showed that some of the compounds possessed strong scavenger activity against hydroxyl radicals and superoxide anion radicals [82]. *In vivo* studies have shown that treating animals with *Rhodiola* prevented free radical-mediated damage and lipid peroxidation by increasing the concentration of GSH and the activity of superoxide dismutase [81]. A human study showed that treating high altitude polycythemia patients with *Rhodiola* increased superoxide dismutase activity in red blood cells and decreased plasma contents of malondialdehyde [83].

Chronic hypoxia has been shown to induce several biological changes in the pancreas. Theses changes may be beneficial to the cellular systems such as preparing the cells to adapt to the new environment. However some changes may be harmful such as the potentiation of free radical formation. *Rhodiola* treatment may prevent hypoxia-induced pancreatic injury in two ways. By increasing the availability of intracellular oxygen and the efficiency of oxygen utilization, it reduces the shortage of the oxygen supply and thus minimizes the changes induced by chronic hypoxia. Alternatively, by its scavenger activity and enhancement of endogenous antioxidant defense, it may reduce oxidative injury induced by chronic hypoxia. However, additional experimental data are required to fully elucidate the mode of action of this drug.

Conclusion

Chronic hypoxia causes an activation of pancreatic RAS including the increased expression of angiotensinogen, AT_{1b} and AT_2 receptors. Consequently, ROS may be produced via the activation of XO or NAD(P)H oxidase. The ROS may cause oxidative damage in the pancreas and other tissues either directly or indirectly via the formation of other radicals such as reactive nitrogen species. *Rhodiola* treatment may protect hypoxia-induced pancreatic injury in two ways. It prevents hypoxia-induced biological changes by increasing intracellular oxygen diffusion and the efficiency of oxygen utilization. Alternatively, it reduces hypoxia-induced oxidative damage by its antioxidant activities. The biological mechanisms involved require further elucidation.

Key words Antioxidants; Medicine, Chinese Traditional; Medicine, Herbal; Oxidants; Pancreas; Pancreatic Diseases; Reactive Oxygen Species; Receptors, Angiotensin

Abbreviations ACE: angiotensin-converting enzyme; GSH: glutathione; GSSG: glutathione disulfide; NO: nitric oxide; RAS: renin-angiotensin system; ROS: reactive oxygen species; XDH: xanthine dehydrogenase; XO: xanthine oxidase

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