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The Study on the Relation between Neutrophil Respiration Burst Activity and Oxidative Tissue Injury during Ischemia-Reperfusion

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Abstract: During the ischemia-reperfusion， ROS are produced in the cells of the ischemic or reperfused tissue or organ by the route of mitochondria, xanthine oxidase, catecholamine-self oxidation, especially by xanthine oxidase route. ROS can activate endothelia cells to release P-Selectin or express de novo a series of CAMs, such as E-Slectin, VCAM and ICAM through inducement of the production of TNF-α or through oxidation-reduction sensitive factors such as NF-KB、AP-1. At the same time endothelia cells also set free chemotactic factors, such as IL-8, MIPs etc, which can activate Integrins on the membrane of Neutrophils. The de novo CAMs that endothelia cells express and activated integrins, such as αLβ2(CD-11a, LFA-1), αMβ2, αXβ2 result in the fixed adhesion of neutrohils to endothelial cells, which at last does much harm to blood vessels and tissues by respiratory burst activity or obstructing the blood vessel. During exercise and recovery period after exercise, the ischemia and reperfusion of tissues or organs also exists, but because of the distinct increase of IL-1ra, sTNF-R, IL-10 and IL-6, the amount of TNF-α in blood is small. What is more, IL-6, IL-8 and especially enzymes released during the course of respiratory burst activity, work together to make CD-11b on the neutrohils shed or down-regulate. so it is evident that oxidative injury caused by exercise-induced ischemia/reperfusion differs from what is caused during the general ischemia/reperfusion, that is to say, oxidative injury caused by exercise-induced ischemia/reperfusion seems to have nothing to do with the neutrophils or their respiratory burst activities.

Keywords: Ischemia/Reperfusion; TNF-α; CD-11b

Swell found the dog, whose coronary artery was ligated for some time, then by releasing the ligation, died because of ventricular fibrillation in 19955. In 1967 Bulkley and Hutchins found the death of the cardiac muscle cells on the patients whom was made Coronary Artery Bypass operation on. Greenberg, in 1981, confirmed the mucous membrane cells of the small intestine were severely injured when reperfused after ischemia for 3 hours. So it is obvious that there exists serious injury accompanying with the reperfusion in the ischemic tissue, the injury may be caused by the neutrophil RBA (respiratory burst activity). Now It is universally acknowledged that the I/R(ischemia/reperfusion) injury is the phenomenon that ischemic tissues or organs lose their normal physiological functions or are suffering a series of injury of structure, but once reperfused, their normal function and structure can not only be rehabilitated but also deteriorate. So the absence of oxygen and nutrients from blood creates a condition in which the restoration of circulation results in

inflammation and oxidative damage through the induction of oxidative stress rather than restoration of normal function.

1. Neutrophil activation and tissue oxidative injury during I/R

When neutrophils phagocytose microbes, its product, ROS(reactive oxygen species) increased remarkably with the augment of oxygen consumption quantity, at the same time, the Pentose Phosphate Pathway is put into use，the whole course is called RBA. Now it has been proved that neutrophils play significant and essential role in the ischemia-reperfusion injury of vascular endothelial cells (1). Blockade of neutrophil elastase is effective in attenuating the clinical and pathological manifestations (2). Under the normal physiological condition, neutrophils and vascular endothelial cells(ECs) do not touch each other. however ,during ischemia-reperfusion (I/R) A variety of pro-inflammatory cytokines, such as TNF-α(tumor necrosis factor), IL-1-β(interleukin), and IL-6 are induced , which promote expression of cell adhesion molecules(CAM) such as selectins on the vascular ECs surface, At the same time ECs also produce chemotactic factors, such as IL-8 and macrophage inflammatory proteins (MIPs; CC chemokine) in responses to ischemia, which effectively recruit neutrophils and monocytes to the site of tissue injury/inflammation. So it goes without saying that it is these cytokines that first mediate the neutrophil rolling adhesion then the firm adhesion of neutrophils to vascular endothelial cells by activating the integrins on the surface of neutrophils. The adhesion, especially the firm one can triggers RBA of the neutrophils.

1.1 Expression and activation of CAMs and adhesion of neutrophils to vascular endothelial cells

Vascular endothelial cells, even under normal physilological condition, can express P selectin which is stored at it's Weibel Palade body. While vascular endothelial cells are activated, P selectin can be transferred to the cell membrane in a few minutes but disappears soon. Activated ECs also express de novo E selectin which is transferred to the outside of the cells after 4-6 hours where it can only exist 24-48 hours, then disappears. P-selectin and L-selectin can both associate with L-selectin on the surface of neutrophils, resulting in the rolling adhesion between ECs and neutrophils. What's more, activated ECs can express de novo a set of integrin-ligands such as intercellular adhesion molecule-1(ICAM-1) and vascular adhesion molecule-1(VCAM-1), both of which are a member of Ig-SF(immunoglobulin-superfamily). But under normal physiological condition, integrins on the surface of neutrophils such as $\alpha_L\beta_2$(CD11a/CD18, LFA-1),$\alpha_M\beta_2$(Mac-1, CD11b /CD18), $\alpha_X\beta_2$(P150,95 CD11c/CD18) are not activated, so it is impossible for them to interact with Ig-SF molecules on the surface of the ECs. Once being activated these integrins engage their counter-ligand such as VCAM-1 and ICAM-1, resulting in firm adhesion to ECs.

1.1.1 TNF-α and the CAMs expression

While some organ or tissue is under the condition of I/R, by the route of mitochondria, xanthine oxidase, catecholamine-self oxidation, especially by xanthine oxidase route, the organ or tissue can produce some amount of ROS, the latter may trigger leukocyte recruitment and inflammation by the following ways. One is through the redox-sensitive transcription factors in the ECs, such as NF-κB (nuclear factor- κB), AP-1(activator protein) which can promote the expression of CAMs, thereby directly triggering vascular inflammation. The other is through inducement of the expression or production of pro-inflammation factors, TNF-α (3),

LPS(lipopolysaccharide) (4, 5), which both can activate ECs. The activation of ECs and subsequent accompanying injury is main reason for the acute or chronic inflammation that takes place during hypoxia (6), athrosclerosis (7), I/R(11) and so on.

Now we focus on the influence of TNF-α on the CAMs expression. The binding of TNF-α to TNF-R1 on the membrane of EC leads to the recruitment of TRADD (TNF-R1 associated death domain proteins) forming the receptor complex. TRADD subsequently recruits other effect proteins into the complex. TRAF2 (TNF receptor associated factor 2) and the death domain kinase RIP(receptor interacting protein) have been shown to interact directly with the TRADD. The interaction can result in the activation of NF-κB. Like other kinds of cells inactive, NF-κB is sequestered in the EC cytoplasm through its interaction with the inhibitory proteins known as IκBs. In response to various stimuli, such as the pro-inflammatory cytokines TNF and IL-1, IκBs are phosphorylated by their kinase, IKK, and are rapidly degraded by the proteasome after polyubiquitination，resulting in NF-κB liberation and its entry into the nucleus, where it activates gene expression. Binding sites for NF-κB have been identified in the promoter regions of the genes for E-selectin, VCAM-1 and ICAM-1(12,13,14), while a binding site for AP-1 has been localized on the promoter region of the ICAM-1 gene. Once NF-κB and AP-1 translocates into the nucleus, ECs are activated and start to express de novo a set of integrin-ligands such as ICAM-1 and VCAM-1(8, 9, 10). So far many measures are being taken to keep NF-κB from transferring into nucleus so that the expression of ECAM (endothelial cell adhesion molecules) are down-regulated significantly (15, 16).

1.1.2 The activation mechanism of integrins on Leukocyte

The integrins on the Leukocytes are inactive under normal physiological condition decreasing the affinity of the integrin family of receptors, unless they receive “inside-out” signals from leukocyte. When a outside stimulation acting on a leukocyte trigger internal changes of the cell, that is to say, a “outside –in” signals are produced, through a series of physiological and biochemistry mechanism a novel “inside-out” signals are formed and in turn, the “inside-out” signals work on the inside part of integrins to bring out the conformational changes of the molecule outside parts which cause total activation of the integrins. The integrin activations increase the affinity of the integrin family of receptors, which then bind to their counter-ligands. Besides, there exist counter-receptors of chemokines on leukocytes, which attribute to GPCR (G protein-coupled receptors) (17).

At the initial stage of I/R emerging TNF-α makes P selectin, which is stored at it’s Weibel Palade body, translocate to the surface of ECs, besides, it can also make ECs expresse de novo E selectin, ICAM-1 and VCAM. Leukocytes express at the tips of their microvilli carbohydrate ligands for the selectins, which bind to the endothelial selectin. But these are low-affinity interaction with a fast off-rate, and they are easily disrupted by the force of the following blood. As a result, the leukocytes detach and bind again and begin to roll along the endothelial surface. The interaction between ECs and neutrophils, though being low-affinity can produce “outside-in” signals in leukocytes, directly or indirectly increasing the affinity of the integrin family of receptors, which then bind to endothelial cell adhesion molecules such as ICAM-1)/CD54 and VCAM-1. Leukocyte integrin affinity is also rapidly increased by “inside-out” signals from leukocyte chemokine receptors triggered by chemokines such as IL-8, produced by ECs and displayed on its surface. With an increase in leukocyte integrin receptor affinity, leukocytes stop rolling and are arrested, with their cytoskeletons being reorganized. It

should noted that the "inside-out" signals also result in alterations in the function of cell junction proteins and/or make the endothelial cells contract, thereby facilitating leukocyte transmigration into the tissue.

It is well-known that epithelial cells constitute a physiologic barrier, while intercelluar junctions, particularly tight junctions (TJ), play an important role in the regulation of the epithelial barrier function. Disruption of tight junctions is a common feature of many inflammatory diseases, and loss of specific tight junction proteins has been shown to be predictive of invasion and metastasis of epithelial cancers**(**59, 60**).** In polarized endothelial cells, the TJ is comprised of a complex network of proteins that encircles the cell at the apical-most portion of the lateral membrane**(**61, 62**)**, immunoglobulin superfamily proteins such as junctional adhesion molecules (JAMs), platelet/endothelial cell adhesion molecule-1(PECAM)are found in the TJ. A study found upon treatment of endothelial cells with TNF-α and interferon-γ, JAM-A dissociates from the junctions and redistributes to the apical surface, making the molecule availabe for leukocyte LFA-1 and conferring upon the molecule a regulatory function for junction integrity**(**63**)**. Recently, PECAM was found to recycle along interendothelial border into segments of the junction, across which leukocytes are actively migrating (64). TNF also can cause ECs to undergo shape changes and basement membrane remodeling, which favors extravasation of the arrested leukocytes.

Lastly, TNF activates ECs to produce vasodilator substances, such as prostacyclin(PGI_2) that can blood vessel constrict, increasing local blood flow and delivering more leukocytes to the site where I/R takes place.

1.2 Tissue or organ injury caused by neutrophils

1.2.1 Capillary vessel injury and activated neutrophils

Firstly, the neutrohils tethered to the ECs can cause the change of hemodynamics. Under normal physiological condition, ECs and neutrohils seldom touch each other, which guarantees the rapid blood flow in Microcirculation blood vessel. When I/R occurs, activated neutrophils with larger volume may obstruct blood vessel by its rolling or being arrested on the luminal side of the endothelium, deteriorating ischemic degree and forming a vicious cycle. Secondly, neutrophils can make diameter of blood vessel shrink by releasing potent constricting blood substance, vessel endothelin and angiotensin , both of which exacerbate I/R injury. Lastly, usually ECs produce the same amount of PGI_2 as the platelets produce thromboxane A_2 (TXA_2), but during the I/R, the balance is broken and the amount of the latter exceeds the former, for a lot of ROS released by the activated neutrophils by the way of RBA, do so much harm to the ECs that one of its product, PGI2, decreases predominantly. The imbalance cause blood vessel to shrink further, exacerbating the ischemic tissues or organs.

1.2.2 Activated neutrophils and other tissues or organ injury.

After diapedisis into a tissue neutrophils start to phagocytose the necrotic cells or tissue, triggering RBA which releases not only A variety of pro-inflammatory factors, such as TNF-α, IL-1-β, and IL-6, TXA_2, leukotriene, but also OFR(oxygen free radical) and all kinds of lysosomal enzyme, for example, myeloperoxidase, metalloprotenase, elastase. All these substance can do great harm to the normal tissues or organs. So the initial site of I/R injury is supposed to be at the endothelial surface, the responses reported are mostly common in various organs and tissues: lung, liver, heart, kidney, brain and skeletal muscles (19).

2. Exercise-induced I/R and oxidative tissue injury

During or after exercise it is a common phenomenon for I / R to take place. During exercise, with the help of neuroregulation and humoral regulation, the blood entering the contracting muscle gets more and more, but that entering liver, kidney, or stomach and other visceras gets less. But after exercise the phenominon is the contrary to the former, that is to say the blood entering the liver, kidney,or stomach and other visceras gets more and more. Some studies (1, 2)find out that in I/R tissue neutrophils RBA is the main way to produce ROS, which cause oxidative tissue injury. But other ones (20, 21, 22, 23, 24)hold opposite viewpoints, affirming Exercise-induced oxidative tissue injury does not attribute to I/R-induced oxidative injury. For example, Nagatomi(19) claimed It was unlikely that the oxidative damage observed in such high-intensity endurance exercise attributed to I/R injury, in which the neutrophil-endothelial interaction played a significant role, unless complications such as severe dehydration or heat stroke are involved.

2.1 The number of neutrophils and its activity and function

In I/R inflammatory injury, lysosomal enzymes such as elastases and myeloperoxidase are released in an explosive manner by degranulation. These enzymes, with some pro-inflammatory and anti-inflammatory factors increasing, also increase after prolonged endurance exercise. But the increase in the enzyme activity in the plasma was proportional to the increase in the number of neutrophils (25, 26). The absence of enzyme activity amplification implies that the enzymes' increase after exercise is not due to increased neutrophils degranulation which usually accompanies RBA. Therefore, it is not likely that neutrophil activation is involved in the oxidative stress of exercise, even when the number of neutrophils may rise several fold compared with the resting state (19).

Thus neutrophilia during and after exercise may not necessarily be related to oxidative tissue or endothelial damage due to neutrophil activation and degranulation. Claudicants with ischemic vascular damage showed elevated plasma neutrophil elastase and up-regulated CD11b expression on neutrophils after exhaustive treadmill exercise. They also exhibited a neutrophilia, but the extent of the neutrophilia was similar to the control subjects. Other studies (27, 28) also find out that high-intensity(HI) exercises increase neutrophil counts. However, The neutrophilia does not appear to influence the release of myeloperoxidase from neutrophils or respiratory burst activity. The increase in neutrophil respiratory burst activity after HI is likely due to the priming effects of growth hormone and IL-6 on the reduced NADP oxidase, a enzyme that is responsible for generating reactive oxygen species in neutrophils (55).

2.2 TNF changes in blood plasma after or during exercise

Some study reported that there were lots of local production of pro-inflammatory cytokines such as IL-1β and TNF-α within the skeletal muscle after eccentric exercise, but evidence is lacking to suggest that muscle is the source of the following anti-inflammatory cytokines, such as IL-1ra, IL-10, and IL-4, which may be produced by mononuclear cells of the immune system (31, 32). So it goes without saying that after eccentric exercise it is within the skeletal muscle that inflammation breaks out. On the contrary, the systemic levels of IL-1β and TNF-α increase only slightly following eccentric exercise. Conversely a stronger systemic anti-inflammatory response occurs following eccentric exercise, as indicated by elevated plasma levels of IL-1ra, IL-10 and soluble TNF-α receptors (33, 34), so it is impossible that systemic inflammation takes place.

Therefore, although pro-inflammatory cytokines are produced within skeletal muscle after eccentric exercise, their release into the circulation appears to be inhibited. The mechanism of this inhibition is not clarified thoroughly at present，however, more and more evidence proved that IL-6 may be involved. IL-6 has been termed an inflammation-responsive "myokine", because it does not directly reduce inflammation and is produced within skeletal muscle (35, 21). Evidence suggests that IL-6 acts indirectly to restrict inflammation by stimulating the production of anti-inflammatory cytokines including IL-1ra, IL-10, cortisol and soluble TNF-α receptors (37). Other data also demonstrate that IL-6 exerts inhibitory effects on TNF-α and IL-1 production. IL-6 inhibits LPS-induced TNF-α production both in cultured human monocytes and in the human monocytic line U937(38), and levels of TNF-α are markedly elevated in anti-IL-6-treated mice and in IL-6 deficient knock-out mice(39,40). In addition, recombinant human IL-6(rhIL-6) infusion as well as exercise inhibits the endotoxin-induced increase in circulating levels of TNF-α in healthy humans (41). Some scholars find out that IL-6 is the first cytokine present in the circulation during exercise and the appearance of IL-6 in the circulation is by far the most obvious and its appearance precedes that of the other cytokines (36). The level of circulating IL-6 increases in an exponential fashion (up to 100 fold) in response to exercise, and decreases in the post-exercise period (42, 43), but the classical pro-inflammatory cytokines, TNF-α and IL-1β, generally do not increase with exercise (36). Research within the past few years have demonstrated that IL-6 mRNA is up-regulated in contracting skeletal muscle (44, 45), and that the transcriptional rate of the IL-6 gene is markedly enhanced by exercise (46). In addition, it has been demonstrated that the IL-6 protein in expressed in contracting muscle fibers (47, 48), and IL-6 is released from skeletal muscle during exercise (49). The study of marathon running indicates, the plasma concentrations of TNF-α (2.3×), IL-1β (2.1×), IL-6 (128×) and IL-10 (27×) are elevated immediately after running, while soluble TNF-α receptors (~2×) and IL-1ra (39×) peak 1–1.5 h later (50). IL-1ra, IL-10 and soluble TNF-α receptors exert their respective anti-inflammatory effects by inhibiting signal transduction via the IL-1 receptor (51), inhibiting cytokine gene expression and production in mononuclear cells (52), binding and neutralizing circulating and membrane-bound TNF-α (53).

2.3 The expression variation of neutrophil surface receptor CD-11b

A datum said that, with a fixed duration, exercise could affect the expression of neutrophil surface receptor CD-11b independent of exercise intensity, for both MI and HI reduced the receptor expression, although the reduction of the receptor expression did not impair neutrophil degranulation or respiratory burst activity(55).

2.3.1 The relation between cytokines and the expression variation of neutrophil surface receptor CD-11b

Decreased neutrophil receptor expression after exercise may be mediated by systemic cytokine release. For the expressions of CD11b and CD35 receptors is enhanced and are directly proportional to the blood plasma levels of IL-6 and IL-8 in patients suffering sepsis (54). In contrast, the expressions of CD11b, CD35, and CD16 is reduced and are inversely proportional to the blood plasma levels of IL-6 and IL-8 in patients who have experienced acute trauma and burns (57) and in individuals suffering from arthritis (58). It should be noted that the mechanism that exercise affects CD-11b expression may be different from that which up-regulates CD-11b in the sepsis, for Jonathan Peake found an inverse correlation between CD16 expression and the plasma concentrations of these two cytokines (55).

2.3.2 The relation between RBA and neutrophil surface receptor

One possibility is that the down-regulation of CD11b and CD35 expression was the result of the cleavage of receptors from the neutrophil cell membrane by proteolytic enzymes, for incubation of neutrophils in vitro with serine or metalloproteinase enzymes similar to those enzymes released during neutrophil degranulation causes the proteolytic degradation of the plasma membrane portion of the CD11b, and CD35 receptors (56). During exercise it is common for exercise-induced muscle damage or Muscle damage resulting from lengthening contractions to take place. Muscle damage attracts leukocytes to the site of injury. Neutrophils firstly invade skeletal muscle within several hours and remain present up to 24 h after exercise, then, macrophages are present in muscle from 24 h to 14 days after exercise .Neutrophils and macrophages contribute to the degradation of damaged muscle tissue by RBA, that is to say, by releasing reactive oxygen, nitrogen species, as well as serine or metalloproteinase enzymes which may penetrate blood vessel wall and enter the blood, touching the neutrophils and down-regulating the expression of CD11b, and CD35. So it is evident that the down-regulation of neutrophil surface receptor may attribute to RBA induced by muscle injury. The large increase in plasma myeloperoxidase concentration that occurred after exercise in Jonathan's study (55) is evidence that exercise induced neutrophil degranulation. Therefore, granular enzymes released from neutrophils during exercise might have been responsible for the observed reduction in receptor expression after exercise.

Thus it can be seen, The shedding of the receptors, on one hand, may serve as an adaptive mechanism for inhibiting further tissue injury caused by excessive inflammatory reactions through preventing the neutrophils adhering to ECs, on the other hand, may also allow neutrophils to extravasate and move into tissues. So it can be said that the shedding CD11b is a key link that keeps the inflammation within certain limit or control it only to a limited extent.

3. Conclusion

During the ischemia-reperfusion，ROS are produced in the cells of the ischemic or reperfused tissue or organ by the route of mitochondria, xanthine oxidase, catecholamine-self oxidation, especially by xanthine oxidase route. ROS can activate endothelia cells to release P-Selectin or express de novo a series of CAMs, such as E-Slectin, VCAM and ICAM through inducement of the production of TNF-α or through oxidation-reduction sensitive factors such as NF-KB、AP-1. At the same time endothelia cells also set free chemotactic factors, such as IL-8, MIPs etc, which can activate Integrins on the membrane of Neutrophils. The de novo CAMs that endothelia cells express and activated integrins, such as αLβ2(CD-11a, LFA-1), αMβ2, αXβ2 result in the fixed adhesion of neutrohils to endothelial cells, which at last does much harm to blood vessels and tissues by respiratory burst activity or obstructing the blood vessel. During exercise and recovery period after exercise, the ischemia and reperfusion of tissues or organs also exists, but because of the distinct increase of IL-1ra, sTNF-R, IL-10 and IL-6, the amount of TNF-α in blood is small. What is more, IL-6, IL-8 and especially enzymes released during the course of respiratory burst activity, work together to make CD-11b on the neutrohils shed or down-regulate. so it is evident that oxidative injury caused by exercise-induced ischemia/reperfusion differs from what is caused during the general ischemia/reperfusion, that is to say, oxidative injury caused by exercise-induced ischemia/reperfusion seems to have nothing to do with the neutrophils or their respiratory burst activities.

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