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REVIEW ARTICLE

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The role of free radicals in cold injuries

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Abstract Cold injury is a tissue trauma produced by exposure to freezing temperatures and even brief exposure to a severely cold and windy environment. Rewarming of frozen tissue is associated with blood reperfusion and the simultaneous generation of free oxygen radicals. In this review is discussed the current understanding of the mechanism of action of free oxygen radicals as related to cold injury during rewarming. Decreased energy stores during ischaemia lead to the accumulation of adenine nucleotides and liberation of free fatty acids due to the breakdown of lipid membranes. On rewarming, free fatty acids are metabolized via cyclo-oxygenase and adenine nucleotides are metabolized via the xanthine oxidase pathway. These may be the source of free oxygen radicals. Leukocytes may also play a major role in the pathogenesis of cold injury. Oxygen radical scavengers, such as superoxide dismutase and catalase, may help to reduce the cold induced injury but their action is limited due to the inability readily to cross the plasma membrane. Lipid soluble antioxidants are likely to be more effective scavengers because of their presence in membranes where peroxidative reactions can be arrested.

Key words Cold injury and rewarming · Oxygen radicals · Lipid peroxidation

Introduction

Peripheral tissues cool more rapidly than the core tissues on exposure to cold. The limbs are generally more susceptible to the development of cold injuries. Both wet and dry cold conditions combined with a series of environmental factors produce local cold injuries. Environmental conditions such as temperature, precipitation and wind together with the activity of the individual, the duration of exposure, amount of protection and level of fitness, all contribute to overall susceptibility to cold injuries (Boswick et al. 1979). Hypoxia has been shown to play an important role in the reduction of blood flow to the extremities and therefore may modulate the cold injury (Durand and Martineua 1971). When the temperature of the extremities falls below approx. 10° C a transient vasodilatation may occur which allows a surge of blood flow to the area in the short-term. However, this phenomenon is followed by severe vasoconstriction leading to a reduction in blood flow to the extremities and tissue hypoxia. Tissue hypoxia may further render the limbs vulnerable to cold injuries. Structural and functional changes in the microvascular system may thus have an important bearing on recovery from cold injuries (Kulka 1965). Cold injury is often asssociated with irreversible cell damage. Cell death due to freezing and thawing of tissues at the time of cold exposure may be the primary cause of tissue death leading to cell necrosis (Weatherly – White et al. 1964).

On prolonged exposure to freezing temperatures, the cells become hyperosmolar and then freeze due to a shift of water from the intracellular to the extracellular space (Gadarowki and Esce 1973). If there is severe freezing, haemoconcentration, vascular stasis and microcirculatory insufficiency leading to tissue necrosis in the frozen part may follow (Bellman and Adams-Ray 1956). Furthermore, a shift of the haemoglobin dissociation curve to lower oxygen partial pressures will prevent the release of oxygen, leading to tissue hypoxia (Iyengar et al. 1990), producing an effect that is similar to ischaemia of the tissue vasculature. Rapid rewarming as a treatment measure for cold injuries and frozen tissues has been prescribed (Mills et al. 1960). Upon rapid rewarming, ischaemia is relieved and reperfusion takes place. Recent studies have indicated that the rewarming of frozen tissues is associated with the generation of oxygen-derived free radicals and may lead to further cellular injury (Iyengar et al.

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1990). The effect is similar to those described to occur during reperfusion of ischaemic tissues (McCord 1985). This review attempts to explain the mechanism of oxygen radical generation during rewarming of frozen and cold injured tissues and the role of various therapeutic measures against cold injury as related to oxygen-derived free radicals.

Classification of cold injuries

Cold injury may be differentiated into the following major categories: non-freezing (wet cold), freezing (dry cold), which are subsequently subdivided into localized and generalized injury (hypothermia). Non-freezing cold injuries include, in ascending order of severity, chillblains, pernio, and trenchfoot (immersion foot). Examples of freezing injury are frostnip, frostbite and high altitude frostbite. Hypothermia comprises whole body cooling, and may terminate in total body freezing. Non-freezing types of cold injury may be defined as tissue trauma produced by prolonged exposure to wet cold at temperatures above freezing (Francis 1984). Freezing cold injury is directly associated with very low temperatures, hypoxia and dehydration. This type of cold injury is very complex and occurs due to a direct freezing effect of cold on the exposed parts and due to anoxia resulting from circulatory insufficiency. Decreased circulation may lead to stasis in the tissue blood vessels and capillaries which may result in thrombosis. Thrombosis further aggravates the hypoxia, invariably leading to necrosis and gangrene demarcated by mummification or drying of the affected parts, all within a period of 2 to 3 weeks (Meryman 1957). If thrombus formation is prevented, the cold induced injuries, such as oedema and tissue loss, can be prevented to a significant extent (Mileski et al. 1993).

Both the severity of cold and duration of exposure are important for determining the magnitude of tissue damage (Manson et al. 1991). The rewarming of cold or frozen tissue exacerbates the cellular injury that has already occurred to some extent during cooling of the tissue due to volume expansion and ice formation. Such injury would also include microcirculation damage due to intravascular thrombosis. However, the significance of the rewarming injury may be obscured in the case of severe cold injuries e.g. frostbite. In such cases, revascularization can be largely inhibited due to vascular stasis and thrombus formation and rewarming injury thus becomes insignificant relative to severe cold injury. This is similar to the situation occurring when a tissue is subjected to severe ischaemic insult and reperfusion does not occur following ischaemia due to the 'non-reflow' phenomenon. Rewarming injury is of particular importance in the case of non-freezing cold injuries, because in such cases the tissues are subjected only to short-term ischaemia (Russell et al. 1993).

Mechanism for free radical formation during ischaemia and rewarming

Role of electron transport chain

In mitochondrial respiration, cytochrome oxidase is involved in reducing the oxygen to water without production of free oxygen radicals. The presence of oxygen at the terminus of the chain favours the maintenance of the carrier system in an oxidized state. During ischaemia, when oxygen supply is limited, the electron transport chain of the inner mitochondrial membrane becomes highly reduced and oxygen radicals may be formed. The ubiquinone-cytochrome *b* region of the electron transport chain is the major site of oxygen radical formation when mitochondria are in a maximally reduced state (Cino and Dalmaestro 1989).

Role of free fatty acid metabolites

Severe ischaemia is associated with failure of ATP-dependent ionic pumps, resulting in the influx of Na^+, Cl^-, Ca^{2+} and efflux of K^+ (Hochachka and Dunn 1983). One of the postulated consequences of calcium influx during ischaemia is the initiation of pathways involved in the breakdown of lipid membrane constituents and the accumulation of free fatty acids. With the onset of ischaemia, a decrease in phosphatidylethanolamine (PE), phosphatidylcholine (PC) and total phospholipids in muscle has been demonstrated to occur (Das et al. 1991a). The exact mechanism for the breakdown of lipid membranes is controversial. In an environment relatively poor in ATP, calcium influx may be responsible for the activation of phospholipase C with consequent breakdown of phospholipids in the cell membrane and the liberation of free fatty acids (Jennings and Reimer 1981). Activated phospholipase may further potentiate an ionic redistribution in the cell and mitochondrial membranes (Kettlecamp et al. 1971). Ischaemia may induce adenosine 3′5′-cyclic monophosphate (cAMP) dependent activation of phospholipase A_2. The cAMP-effect is increased by the release of K^+, adenosine and catecholamines, all of which increase with ischaemia (Traystman et al. 1991). Ischaemia has been shown to lead to a rapid increase in free fatty acids and an increase in arachidonic acid (Das et al. 1991a). Arachidonic acid readily intercalates into membranes and produces changes in the packing of lipid molecules (Klausner et al. 1980).

During rewarming, blood flow returns to the ischaemic tissues, and although necessary for tissue recovery, may also lead to additional tissue injuries. During the re-establishment of the circulation there is rapid utilization of free fatty acids, in particular, arachidonic acid. Arachidonic acid accumulated during ischaemia is metabolized upon rewarming via the lipoxygenase and cyclo-oxygenase pathways and has been shown to produce

prostaglandins, thromboxanes and superoxide (Hamberg et al. 1975). The prostaglandins may be responsible for vascular sludging and altered vascular reactivity (Harlan and Harken 1981) and oxygen radicals may cause cold injury directly by lipid peroxidation as evidenced by an increase in malonaldehyde generation (Iyengar et al. 1990). The metabolites of thromboxane and prostacyclin from the cold injured tissue increase significantly in blister fluid presumably because of cellular and muscle damage (Robson and Heggers 1981), indicating the generation of eicosanoids.

Role of purine metabolites

Purine metabolites may also produce oxygen radicals during rewarming. During ischaemia, nucleotides are metabolized to nucleosides and purine bases and a rapid rise occurs in the interstitial concentration of adenosine and hypoxanthine (Seisjo 1981). When the energy charge of a hypoxic cell drops below a certain level, biochemical changes occur that set in motion the destructive production of superoxide anions when oxygen again becomes available in the post-ischaemic phase. During the rewarming of frozen tissue, in the presence of oxygen, hypoxanthine may be metabolized via the xanthine oxidase pathway to produce oxygen radicals (Granger et al. 1981). During ischaemia, there is an increase in the conversion of xanthine dehydrogenase to xanthine oxidase by Ca^{2+} activation (Roy and McCord 1983). Rosen and Freeman (1984) have reported the direct detection of superoxide generation by endothelial cells after anoxia. Endothelial cells are ubiquitous, being located at the blood tissue barrier, and have been proposed as the initial site of tissue injury. Moreover, endothelial cells are a rich source of the superoxide-generating enzyme, xanthine oxidase (Jarasch et al. 1986). These cells, therefore, may be important for superoxide generation during the rewarming phase.

Role of leukocytes

Leukocytes may be directly associated with reperfusion or rewarming injury. Cold injuries lead to inhibition of blood flow and it has been shown that leukocytes may be trapped in the microvasculature (Bourne et al. 1986). During rewarming, leukocytes interact with platelets to metabolize arachidonic acid and may produce eicosanoids. Leucocyte accumulation may alter the distribution of post-ischaemic blood flow by the mechanical obstruction of capillaries as well as by the generation of oxygen radicals via the NADPH-oxidase system similar to that occurring during reperfusion of other ischaemic tissues (Weiss 1986). Ischaemic swelling of leukocytes may also contribute to the non-reflow phenomenon and increased muscular permeability in post-ischaemic skeletal muscle (Strock and Majno 1969). The activated neutrophils may damage endothelial cells and increase endothelial cell monolayer permeability through the production of a series of active oxygen species including superoxide, hydrogen peroxide, and hydroxyl radicals (Serhan et al. 1982). These are capable of initiating peroxidation of membrane lipid components and may lead to changes in membrane fluidity and permeability, protein degradation and ultimately cell lysis. Reperfusion with leucocyte depleted blood has been shown largely to prevent the increase in vascular permeability and resistance suggesting that leukocytes may play a major role in the pathogenesis of ischaemia reperfusion injury in skeletal muscle (Granger et al. 1986).

Role of Ca^{2+} in the formation of oxygen radicals

Ca^{2+} ions may play an important role in the generation of oxygen radicals in several pathways. An influx of Ca^{2+} ions, promoted by a Ca^{2+} ionophore, and activation of protein kinase C have synergistic effects on superoxide release by endothelial cells (Matsubara and Ziff 1986). During ischaemia an increase in intracellular calcium has been observed,which activated phospholipases and liberated free fatty acids particularly arachidonic acid (Das et al. 1991a). During rewarming, arachidonic acid may be metabolized via the lipoxygenase and cyclooxygenase pathways and therefore may be the source of oxygen radicals and prostanoids. Ca^{2+} ions may play a major role in activation of the protease, calpin, which cleaves a peptide bond in xanthine dehydrogenase to form xanthine oxidase in the endothelium of blood vessels (McCord 1985). Elevated xanthine oxidase activity during reperfusion may contribute to oxygen radical formation as a result of the metabolism of xanthine to uric acid.

Mechanism of cold injury by free radicals

The precise mechanism by which oxygen-derived free radicals and their metabolites cause cell injury is not clear. However, experimental evidence suggests that lipid peroxidation reactions on the cell membrane may play an important role in radical-mediated cell injury. For example, lipid peroxidation of biological membranes has been shown to lead to structural alterations and abnormal membrane function (Chow 1979). The mechanism of free radical-mediated lipid peroxidation involves at least three distinct phases. The initiation step occurs when a free radical (e.g. OH^-, O_2^-) interacts with a polyunsaturated fatty acid (PUFA) and extracts a proton, forming a fatty acid radical. This step is followed by the second or propagation phase, in which the fatty acid radical can react with oxygen, generating a fatty acid peroxyl radical. The fatty acid peroxyl radical can react with other lipids and proteins, perpetuating the transfer of protons with subsequent oxidation of substrates.

Therapeutic efficacy of radical scavengers and anti-oxidants

In the light of recent knowledge, various radical scavengers and anti-oxidants have been suggested to be effective in preventing biochemical, histological and physiological abnormalities after transient ischaemia (Paton 1987). The enzymes superoxide dismutase (SOD) and catalase may function to reduce cold-induced cell injuries. The role of SOD is to rapidly dismutate superoxide to form oxygen and hydrogen peroxide, whereas catalase very effectively breaks down hydrogen peroxide to oxygen and water. In addition to preventing the direct toxicity of superoxide, SOD may also decrease the amount of substrate available for the Fenton reaction and formation of the peroxy nitrite anion (Beckman et al. 1990). Intravenous administration of SOD and catalase has been shown to support tissue recovery from cold injury (Das et al. 1991b). However, the enzyme SOD has two drawbacks as a therapeutic agent. Firstly, it is readily excreted and has a circulatory half-life of only 8 min in rats (Turrens et al. 1984). Secondly, copper-zinc containing SOD is a large water-soluble molecule and therefore cannot readily penetrate cell membranes (Beckman et al. 1988).

Perhaps the most widely acknowledged antioxidant in biological systems is α-tocopherol or vitamin E. This lipid soluble molecule functions as a free radical scavenger and plays an important role in protecting and maintaining the integrity of cell membranes against lipid peroxidation (Tappel 1972). Administration of vitamin E, before the onset of ischaemia, attenuates lipid peroxidation during reperfusion in the brain (Yamamoto et al. 1983). A deficiency of vitamin E in animals is manifest as increased susceptibility to free radical oxidative injury following ischaemia/reperfusion. Therefore, vitamin E may be used as a therapeutic agent against cold injury, due to its ability to scavenge oxygen radicals (unpublished observations of Bhaumik G and Purkayastha SS).

The use of another antioxidant as a therapeutic agent against cold injury, namely vitamin C or ascorbic acid, has been demonstrated (Purkayastha and Mathew 1992; Purkayastha et al. 1993). As a major water-soluble antioxidant, vitamin C is capable of maintaining sulphydryl compounds in a reduced state, participating in many redox reactions and scavenging singlet oxygen and free radicals (Bendich et al. 1986). Vitamin C may also be involved in regeneration and restoring the antioxidant properties of vitamin E (Chen and Chang 1979). Hruba et al. (1982) have shown in guinea-pigs that a diet marginally deficient in vitamin C resulted in lower vitamin E levels in some tissues (i.e. lungs and liver) compared to those receiving adequate vitamin C. Vitamin C has also been shown to enhance iron absorption and produce intracellular stabilization of iron-binding proteins including ferritin. Thus the therapeutic efficacy of vitamin C against cold injury may be attributed to its antioxidant properties and its role in the regeneration of vitamin E. In conclusion, further studies are required definitively to demonstrate radical-mediated injury during rewarming. The action of free radical scavengers and antioxidants in reducing cold injuries by inhibiting the formation of free radicals should also be confirmed.

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