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VASCULAR REACTIVITY FOLLOWING ISCHEMIA/REPERFUSION

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1. ABSTRACT

Data in the literature supports the hypothesis that reactive oxygen species generated in the vascular wall alter vascular regulation. At present the majority of the literature tends to suggest that oxidant induced damage on the smooth muscle cell impair vasoconstriction. However, direct action of oxidants on the smooth muscle cell impair vasoconstrictor function. Differences in studies in the literature are likely to be reconciled when the target sites of reactive oxygen species are considered. Future research in this area should lead to a more comprehensive understanding on the impact of these pathways on vasoregulation in postischemic tissue.

2. INTRODUCTION

Numerous studies in recent years have provided evidence that periods of tissue ischemia followed by reperfusion lead to both vascular and parenchymal cell damage. From these reports, it has become universally accepted that the reintroduction of oxygen during reperfusion fuels the generation of reactive oxygen metabolites that are capable of producing organ as well as vascular dysfunction. The oxidant burst that accompanies reperfusion can directly damage endothelial and vascular smooth muscle cells leading to increased capillary permeability and alterations in vascular resistance. The importance of the vasculature in reperfusion injury is exemplified by the fact that preservation of vascular function during the reperfusion period often preserves organ function. The purpose of the present review is to summarize current evidence linking reactive oxygen species to alteration in vascular smooth muscle function. It is beyond the scope of this review to discuss the alterations in endothelial cell function as it relates to capillary permeability.

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3. MECHANISM OF VASCULAR CONTRACTION AND RELAXATION

The physiological role of the resistance vasculature in the regulation of organ blood flow is well documented. In general, factors that relax vascular smooth muscle increase blood supply while factors that contract vascular smooth muscle reduce organ perfusion. The balance between vascular relaxation (vasodilation) and vascular contraction (vasoconstriction) is carefully maintained in the resting organ such that resistance to blood flow and organ perfusion are maintained relatively constant. Any disturbance that alters this balance raises or lowers organ blood flow. Inasmuch as the overall objective of this review is to summarize the role of oxidants in postischemic vascular dysfunction, we believe that a brief discussion of the cellular events mediating vascular smooth muscle contraction and relaxation is needed.

3.1 Cellular mechanism of receptor mediated vasoconstriction in normal blood vessels

The coupling of vasoconstrictor agonists with membrane receptors is known to involve activation of membrane-associated guanosine, 5'-triphosphate (GTP) binding proteins, stimulation of phosphatidyl inositol turnover and activation of protein kinase C. These events can lead to the opening of calcium channels on the cell membrane and/or sarcoplasmic reticulum. L-type calcium channels on the sarcolemma are activated by voltage as well as phosphorylation. Calcium release channels

on the sarcoplasmic reticulum predominately respond to elevation in inositol trisphosphate (IP_3). Calcium binding to calmodulin leads to the activation of myosin light chain kinase, phosphorylation of the myosin head and actomyosin interactions. In addition to this well defined calcium dependent pathway, recent reports indicate that protein kinase C activation can promote vascular smooth muscle contraction in the absence of increased cytosolic calcium levels. This latter "PKC-dependent, Ca^{2+}-independent" mechanism has been postulated to involve a change in calmodulin affinity such that calmodulin-calcium binding occurs at the normally low cytosolic calcium concentrations. Both calcium dependent and calcium independent protein kinase C pathways require G-protein activation (1-4).

Perhaps the single most important step in smooth muscle tension generation involves the phosphorylation of the myosin light chain. It is generally accepted that dephosphorylated myosin does not couple with actin to any significant degree. Phosphorylation of myosin by a calmodulin dependent kinase increases the affinity of myosin, thereby promoting cross bridge formation. Tension generation and muscle shortening are the consequence. Dephosphorylation of myosin by phosphatases are required to return actin and myosin by phosphatases are required to return actin and myosin to their normal low affinity states.

3.2 Mechanism of vasodilation in normal blood vessels

The mechanism of vascular smooth muscle relaxation has also been the subject of many previous reports. It is now generally accepted that relaxation of vascular smooth muscle from a resting level involves the lowering of intracellular calcium. Receptor operated vasodilation has been shown to be mediated by both cAMP and cGMP dependent pathways.

3.3 Cellular mechanism of cAMP dependent vasodilation

Two pathways have been suggested whereby cAMP can relax vascular smooth muscle: 1) decreasing intracellular calcium and 2) decreasing the rate of myosin phosphorylation. Adelstein and coworkers (5) have demonstrated that smooth muscle myosin light chain kinase can be phosphorylated by a cAMP-dependent protein kinase (Protein Kinase A). *In vitro* studies indicate that phosphorylation of myosin kinase via a cAMP dependent pathway decreases the affinity of the myosin kinase for the calcium-calmodulin kinase that is necessary for the phosphorylation of myosin. The result is a decreased calcium sensitivity of the smooth muscle contractile machinery. Subsequent studies by Kimura *et al.* (6) demonstrated that cAMP enhanced calcium pump activity. This led to the suggestion that cAMP promotes vasodilation by decreasing intracellular calcium. It is also known that the sarcoplasmic reticulum calcium pump was sensitive to stimulation by cAMP, the plasma membrane pump was not. Several other laboratories have shown that cAMP dependent protein kinases phosphorylate and prevent the opening of dihydropyridine sensitive calcium channels. This latter action limits the rise in calcium in response to vasoconstrictor agonists. Collectively, the aforementioned data suggests that decreased myosin phosphorylation and/or decreased intracellular calcium mediate cAMP dependent vasodilation.

McDaniel *et al.* (7) conducted studies to more precisely define the relative importance of decreased phosphorylation and decreased calcium mobilization as mediators of vascular smooth muscle relaxation in normal pig carotid artery. These investigators reported that forskolin induced increases in cAMP reduced intracellular calcium, myosin phosphorylation and stress in vascular tissues precontracted with phenylephrine or histamine. The reduced contractile tension more closely correlated with the reduction in intracellular calcium, not the calcium dependence of myosin light chain phosphorylation. As a result, these investigators concluded that cAMP induced relaxation of agonist stimulated swine carotid artery is primarily related to cAMP mediated reductions in myoplasmic calcium.

3.4 Mechanism of cGMP dependent vasodilation

Several studies in recent years have documented the fact that increases in intracellular cGMP relax vascular smooth muscle (8). The cellular actions of cGMP appear to largely be mediated by cGMP dependent kinases . Reports in vascular smooth muscle indicate that cGMP dependent kinases depress the rise in cytosolic calcium that occurs in response to vasoconstrictor agonists such as angiotensin II. The exact mechanism by which cGMP limits calcium influx remains unclear, but it has been suggested that inhibition of dihydropyridine sensitive channels may be involved. A second postulated action of cGMP on smooth muscle is via Ca^{2+}-ATPase activity can be increased by cGMP dependent protein kinases. Whether this action is due to direct phosphorylation and activation of the pump or indirect activation through phosphorylation of a regulatory protein remains unclear. Nonetheless, the net effect of increased cGMP dependent kinase is to enhance calcium pump activity. The combined actions of decreased calcium influx and increased calcium pump activity lower intracellular calcium and mediate cGMP dependent relaxation.

Recently, Lincoln *et al.* (8, 9) have suggested a possible link between cGMP and cAMP dependent vasodilator pathways. Studies in primary

vascular smooth muscle cell cultures indicated that forskolin increased cAMP levels, produced slight reductions in basal cytosolic calcium levels, and attenuated the rise in intracellular calcium produced by vasopressin. Passaged vascular smooth muscle cells in the same study exhibited a cAMP dependent increase in intracellular calcium, a somewhat atypical response for a vasodilator stimuli. Introduction of cGMP dependent kinase and forskolin into the passaged smooth muscle cells led to a decrease in calcium. As a result, Lincoln *et al.* (8, 9) suggested that "activation of cGMP dependent protein kinase in response to forskolin was responsible in part at least for the decrease in Ca^{2+} levels."

4. REACTIVE OXYGEN METABOLITES IN ISCHEMIA/REPERFUSION

The idea that periods of organ ischemia following by reperfusion lead to the generation of reactive oxygen metabolites has gained wide acceptance since it was first proposed. Early experimental evidence linking free radicals to ischemic vascular injury was provided by Granger *et al.* (10) in the feline intestine. According to their original hypothesis, periods of ischemia lead to degradation of ATP to AMP which is further catabolized to hypoxanthine. The low oxygen tension during the ischemic period was postulated to also lead to the conversion of xanthine dehydrogenase to xanthine oxidase. The reintroduction of molecular oxygen upon reperfusion of the ischemic intestine was postulated to lead to the generation of superoxide from a xanthine oxidase catalyzed reaction of hypoxanthine with oxygen. Since this sentinel publication, the mechanisms whereby superoxide leads to vascular dysfunction have been widely studied. According to current literature, superoxide can be formed from the interaction of hypoxanthine with molecular oxygen in a reaction catalyzed by xanthine oxidase. In the presence of superoxide dismutase and water, superoxide is converted to hydrogen peroxide. Catalase can degrade hydrogen peroxide to molecular oxygen and water. In the absence of catalase, hydrogen peroxide forms hydroxyl radicals. In addition to this pathway, Beckman and coworkers (11) have suggested that nitric oxide is capable of interacting with superoxide to form the highly reactive peroxynitrite. Peroxynitrite can subsequently be converted to perhydroxy nitrite and ultimately hydroxyl radicals. Superoxide, peroxynitrite and hydroxyl radicals are all potent oxidants with cytotoxic properties.

Although it is conceivable that oxidants generated immediately upon reperfusion can lead to vascular dysfunction the exact source of these oxidants have remained somewhat elusive. If one is to suggest that xanthine oxidase derived free radicals are involved then evidence that vascular smooth muscle contains the necessary metabolic machinery to generate reactive oxygen metabolites is required. Support of this contention was provided by Suzuki and Ford (12) who measured xanthine oxidase levels in postnuclear supernatant of bovine aortic smooth muscle. Levels of approximately 1.2 mU/g wet weight (≈ 5 mU/ml) were detected. As a result of these data it was suggested that the generation of superoxide via a xanthine oxidase catalyzed reaction could be responsible for vascular smooth muscle damage in certain pathophysiological conditions. Subsequent data by Gao *et al.* (13) in endothelial denuded rat aorta demonstrated that periods of hypoxia followed by reoxygenation led to an oxidant mediated reduction in vasoconstrictor effectiveness. In view of the aforementioned data, one can only conclude that reactive oxygen species generated in the smooth muscle cell are capable of modulating vascular function. In the sections which follow, we will focus on the data supporting a role of oxidant mediated alterations in vasoconstrictor and vasodilator function in ischemia /reperfusion as well as hypoxia/reoxygenation.

To date, few studies have assessed oxidant induced alterations in smooth muscle function following ischemia reperfusion of the intact organ. One such study from our laboratory evaluated the effects of 10 min ischemia and 90 min reperfusion on vascular norepinephrine responsiveness in the rat small intestine (14). Ischemia/reperfusion produced a significant increase in the EC_{50} concentration of norepinephrine, factors consistent with reduced vasoconstrictor effectiveness. Superoxide dismutase prevented the loss of vasoconstrictor function associated with ischemia/reperfusion. Pretreatment with catalase or deferoxamine did not prevent the loss of vasoconstrictor function. These data led us to conclude that alterations in vasoconstrictor function were mediated by the superoxide anion. Although these observations were of interest, they did not provide detailed information regarding the nature of the vascular smooth muscle vasoconstrictor deficit.

The use of isolated vascular preparations have provided the most insight into free radical induced alterations in vascular contractile function. Wolin and Belloni (15) evaluated the ability of reactive oxygen metabolites to impair vascular smooth muscle responsiveness to alpha-adrenergic stimuli, *in vivo*. These investigators demonstrated that micromolar quantities of exogenously generated reactive oxygen species (via xanthine oxidase) interfere with the vasoconstrictor actions of norepinephrine, but not phenylephrine. Introduction of superoxide dismutase into the bathing solution prevented the oxidant-induced decrease in vascular norepinephrine responsiveness. Catalase did not

protect in this study. These investigators concluded that the observed reductions in norepinephrine sensitivity produced by exogenously generated oxidants were due to the superoxide anion. The experimental design used by Wolin and Belloni (15) did not allow for dissociation of agonist oxidation from vascular smooth muscle damage. As such, the superoxide-induced decrease in tension may have been largely due to inactivation of norepinephrine.

The vasoactive properties of reactive oxygen metabolites generated by electrical field stimulation were studied by Lamb and Webb (16). These investigators also demonstrated that reactive oxygen species interfere with norepinephrine induced vasoconstriction, *in vitro*. However, unlike the study of Wolin and Belloni (15), Lamb and Webb (16) were able to prevent the decreased contractile function of vascular smooth muscle with catalase. Superoxide dismutase was also found to be protective. As a result, it was proposed that reactive oxygen metabolites including the superoxide anion, hydrogen peroxide and/ or secondarily derived free radicals, are capable of mediating vascular smooth muscle dysfunction. The reduced vascular contractile function was attributed to both direct destruction (oxidation) of norepinephrine as well as a direct action of reactive oxygen metabolites to inhibit vascular smooth muscle contraction. The latter conclusion was based on the observation that reactive oxygen metabolites relax vascular smooth muscle in pharmacologically denervated (alpha + beta blocked) strips of dog coronary artery subjected to electrical field stimulation.

Recent studies from our laboratory (17) have assessed the ability of exogenously derived free radicals to modulate vascular contractility, *in vitro* . Incubation of rat aortic rings with xanthine/xanthine oxidase for 30 min. produced a significant reduction in vascular responsiveness to norepinephrine, even after the vessel baths had been rinsed free of the oxidant generating solution. The observed vasoconstrictor deficit could not be attenuated by endothelial denudation, suggesting that oxidants generated by the interaction of superoxide with nitric oxide were not major contributors to the vasoconstrictor dysfunction. Addition of superoxide dismutase or catalase to the vessel bath only partially prevented the oxidant mediated vascular injury. However, the combination of superoxide dismutase and catalase completely preserved vascular contractile function. The results of this study were in direct contrast to previous studies of Wolin and Belloni (15), in which oxidation of norepinephrine could not be ruled out. Inasmuch as the vessel rings were studied after the oxidant generating system had been thoroughly rinsed from the bath, our data suggested that direct smooth muscle injury, not oxidation of the vasoconstrictor explained the reduced vascular contractile responses.

Although the aforementioned studies provide evidence in support of oxidant mediated reductions in vasoconstrictor function, there is also evidence to suggest that the converse can also occur. Rhoades *et al.* (18) evaluated the effects of exogenously generated oxidants on pulmonary artery contraction, *in vitro*. These investigators reported a significant increase in tension generation of KCl precontracted pulmonary arteries following exposure to reactive oxygen species. The enhanced contraction could be prevented by antioxidants. Several possible mechanisms have been proposed to explain the enhanced contractile properties of preconstricted vascular preparations exposed to oxidants. One such hypothesis is that oxidant induced endothelial damage reduces basal production of nitric oxide thereby enhancing vasoconstrictor effectiveness (19, 20). In a recent study from our laboratory, Yokoyama *et al.* (21) demonstrated that NO release is decreased following hypoxia/reoxygenation, *in vitro* . The reduced nitric oxide release was attributed to endothelial cell death as evidenced by propidium iodide staining of a significant portion of endothelial cells. Studies by Katusic *et al.* (22) suggest that release of constrictors from endothelial cells enhance vasoconstriction in the presence of exogenously generated oxidants. These investigators demonstrated that xanthine/xanthine oxidase and hydrogen peroxide stimulated the production of 6 keto-prostaglandin $F_{1\alpha}$, prostaglandin-$F_{2\alpha}$, prostaglandin-E_2 and thromboxane-B_2 by endothelial cells. Vasoconstriction resulted from the enhanced prostaglandin and thromboxane release. These authors concluded that both enhanced endothelial metabolism of arachidonic acid products and superoxide mediated destruction of nitric oxide mediated the increased contraction of the basilar artery.

The aforementioned data suggest that exogenously generated reactive oxygen species both increase and decrease contraction in isolated blood vessels. Differences in enhanced and reduced vasoconstrictor responses may be related to the concentrations of reactive oxygen species that the vessels were subjected to. The study by Gao *et al.* (17) from our laboratory used concentrations of xanthine (0.5 mM) and xanthine oxidase (0.1 U/ml) that were approximately 1/10th of that used in the other reports. Although untested in any single study, one is tempted to speculate the data that the low levels of oxidants impair and high levels of oxidants enhance contractile function. Perhaps future studies designed to systematically test this hypothesis are needed.

5. ALTERATIONS IN VASOCONSTRICTOR FUNCTION ASSOCIATED WITH ISCHEMIA/REPERFUSION

The ability of vascular smooth muscle to function after periods of ischemia and reperfusion has been addressed in several studies in recent years. From these reports it is now accepted that ischemia and reperfusion leads to oxidant mediated vascular smooth muscle dysfunction. As such, both increases and decreases in vascular tone have been observed. In spite of the fact that oxidants mediate the vascular dysfunction, the source of oxidants and cellular pathophysiology remain controversial. For example, many investigators believe that oxidants generated in the parenchymal tissue were responsible. Others contend that activation of inflammatory cells and subsequent release of oxidants contribute to the vascular injury. However many studies failed to consider the possibility that reactive oxygen species generated in the vascular smooth muscle cell produced the injury. In order to separate parenchymal and inflammatory cell oxidants from vascularly derived oxidants, we developed an *in vitro* model of hypoxia/reoxygenation that would simulate the changes in oxygen tension associated with ischemia/reperfusion. The first of these studies examined the effects of 15 min hypoxia (PO2 < 5 torr) and 30 min reoxygenation (PO2 approximately 500 torr) on vascular norepinephrine responses in rat aorta (23). The results of these studies indicated that brief periods of hypoxia followed by reoxygenation significantly impaired aortic constrictor responses to norepinephrine. Incubation of the aortic rings with superoxide dismutase and catalase completely preserved contractile function. More importantly, neither endothelial denudation nor NOS inhibition by L-NAME protected the vasculature against this oxidant mediated injury. As such we concluded that oxidants derived from the vascular smooth muscle cells were responsible for the impaired adrenergic function in aortic rings. The second aspect of this study involved a systematic analysis of the cellular events associated with adrenoceptor mediated vasoconstriction. Using conventional pharmacological methods we evaluated the effects of hypoxia/reoxygenation on alpha-adrenoceptor affinity. The dissociation constant for norepinephrine was significantly increased while fractional receptor occupancy was unaltered after H/R. These data were consistent with a decreased receptor affinity for norepinephrine and suggestive of a signal transduction defect. Analysis of the cellular events associated with norepinephrine induced vascular smooth muscle contraction suggested that H/R impaired the release of calcium from the sarcoplasmic reticulum.

Subsequent studies from our laboratory examined the effects of H/R on norepinephrine induced vasoconstriction in small mesenteric resistance arteries (< 200 μm in diameter). The purpose of this study was to determine if the altered norepinephrine response was due to impairment of the alpha-1a or alpha-1b receptor pathways. Pharmacological studies had previously demonstrated that alpha-1a receptors rely on the influx of calcium from the extracellular compartment while alpha-1b receptors promoted the release of calcium from the sarcoplasmic reticulum (24). Both subtypes are known to be activated during smooth adrenergic vasoconstriction of resistance arteries. Using an isolated vessel preparation we were able to demonstrate that H/R selectively reduced alpha-1b responses (25). As with our studies in larger vessels these alterations could be prevented by the antioxidants, superoxide dismutase and catalase. These findings supported our contention that release of calcium from the sarcoplasmic reticulum constitutes the major defect in H/R induced vasoconstrictor dysfunction. Direct visualization of calcium transients in FLUO-3 loaded preparations of resistance arteries have provided support of this contention (Gao & Benoit, Unpublished Observation).

6. ACKNOWLEDGMENT

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