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Forum Review

Oxidative Stress and Adaptation of the Infant Heart to Hypoxia and Ischemia

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ABSTRACT

The potential contribution of oxidative stress to cardioprotection in infants induced by adaptation to chronic hypoxia and by ischemic preconditioning is poorly understood. Under conditions of oxidative stress, reactive oxygen species and reactive nitrogen species may contribute to phenotypic changes in hearts adapted to chronic hypoxia and to the pathogenesis of myocardial injury during both ischemia/reperfusion and hypoxia/reoxygenation. Hearts from infant rabbits normoxic from birth can be preconditioned by brief periods of ischemia. In contrast, hearts from infant rabbits adapted to hypoxia from birth appear resistant to ischemic preconditioning. Chronically hypoxic infant rabbit hearts are already resistant to ischemia compared with age-matched normoxic controls, and thus additional cardioprotection by ischemic preconditioning may not be possible. Endothelial nitric oxide synthase (NOS3) protein and its product nitric oxide are increased, but not NOS3 message, in chronically hypoxic infant hearts to protect against ischemia. Chronic hypoxia from birth also increases cardioprotection of infant hearts by increasing association of heat shock protein 90 with NOS3. Normoxic infant hearts also generate more superoxide by an N^{ω}-nitro-L-arginine methyl ester-inhibitable mechanism than chronically hypoxic hearts. Thus, NOS3 appears to be critically important in adaptation of infant hearts to chronic hypoxia and in resistance to subsequent ischemia by regulating the production of reactive oxygen and nitrogen species. *Antioxid. Redox Signal.* 6, 423–429.

INTRODUCTION

CONGENITAL HEART DEFECTS occur in one of every 125 newborn children. One third of these children require a major surgical procedure within the first year of life to prevent premature death. Many of these children exhibit varying degrees of cyanosis where the myocardium is chronically perfused with hypoxic blood. Understanding the mechanisms by which cyanotic congenital heart defects modify the myocardium may provide insights into developing treatments to protect the hearts of these children during corrective surgery.

Adaptation to chronic hypoxia and brief periods of ischemia prior to a sustained ischemic insult protects the myocardium against injury. This chapter addresses the potential contribution of oxidative stress to cardioprotection induced by adaptation to chronic hypoxia and by ischemic preconditioning. Oxidative stress occurs when the production of free radical species and other oxidative molecules exceeds the capacity of the body's antioxidant defenses to detoxify them. Thus, chronic hypoxia associated with cyanotic birth defects may represent a form of oxidative stress. Under conditions of oxidative stress, reactive oxygen species (ROS) and reactive nitrogen species (RNS) may contribute to phenotypic changes in hearts adapted to chronic hypoxia and to the pathogenesis of myocardial injury during both ischemia/reperfusion and hypoxia/reoxygenation.

CYANOTIC BIRTH DEFECTS AND OXIDATIVE STRESS

To investigate the effects of chronic hypoxia from birth on signal transduction pathways and resistance to ischemia, a reproducible, nonsurgical model of cyanosis from birth has been

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developed by this laboratory whereby the myocardium is chronically perfused with hypoxic blood (3). Our model has proven to simulate the essential characteristics of cyanotic congenital heart disease. The model is characterized by decreased arterial oxygen levels, polycythemia, right ventricular hypertrophy, decreased weight gain, and overall failure to thrive (3), similar to what is seen in children with cyanotic congenital heart defects (30). The resulting changes in physiology and biochemistry of the heart are achieved without surgical manipulation. No model exists that reproduces all the characteristics of cyanotic congenital heart defects. This unique model facilitates study of the mechanism of tolerance of hypoxic myocardium to ischemia. Although induction of hypoxia by lowering the concentration of inspired oxygen cannot be equated with the clinical situation of intracardiac shunting, it is the best model to date. It allows us to examine the mechanisms by which hypoxia from birth alters the basic biochemistry and physiology of the myocardium to tolerate subsequent surgical ischemia. Using this model, we showed that adaptation to chronic hypoxia in infant rabbits confers cardioprotection against subsequent ischemia (3).

The relative expression of the isoforms of lactate dehydrogenase (LDH) is regulated in part by the ambient oxygen concentration in the myocardial cell (22, 28, 50). LDH is a tetrameric molecule made up of two different subunits, heart type "H" (LDH_1) and muscle type "M" (LDH_5). The M subunit facilitates the reduction of pyruvate to lactate and reflects anaerobic glycolytic capacity; the H subunit favors oxidation of lactate to pyruvate and reflects aerobic glycolytic activity (39). Exploiting this phenomenon, we determined the relative fractions of the heart and muscle isoforms of LDH expressed in the free wall of the left ventricle of normoxic and hypoxic neonates and found that intracellular levels of oxygen were reduced in hearts from hypoxic animals. Ventricles from hypoxic animals exhibited a relative increase in the expression of the M(LDH_5) isoform and a relative decrease in the expression of the H(LDH_1) isoform of LDH in comparison with normoxic ventricles. The increased fraction of the M(LDH_5) isoform may reflect an increased dependency on anaerobic means of energy production in chronically hypoxic hearts. These changes indicated that in hypoxic infants, despite elevated hemoglobin and hematocrit levels to increase oxygen-carrying capacity, oxygen delivery to the heart was inadequate, resulting in intracellular myocardial hypoxia (3).

REOXYGENATION INJURY IN THE INFANT HEART

Surgical correction of congenital cyanotic defects is performed with the use of cardiopulmonary bypass in early infancy with increasing frequency. Postoperative cardiac dysfunction is the major cause of morbidity and mortality despite successful surgical correction (4). The conventional method of starting cardiopulmonary bypass in infants with preexisting hypoxia is to elevate oxygen tension to ~400 mm Hg. This maneuver may result in the creation of an oxidative stress to the heart. Buckberg has shown that reoxygenation of acutely hypoxic myocardium results in cardiac dysfunction and oxidant injury related to nitric oxide (NO) generation (43). In these studies, the role of NO in generating the hydroxyl radical via peroxynitrite has been demonstrated in an *in situ* blood-perfused piglet heart subjected to acute hypoxia followed by reoxygenation (43). Two hours of severe hypoxia (PaO_2 = 20–30 mm Hg) followed by reoxygenation (PaO_2 > 400 mm Hg) elevated coronary sinus NO levels by 44% of control values. However, in hearts reoxygenated in the presence of the NO synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME), there was no elevation of NO levels above control values. Recovery of posthypoxic contractile function was also improved in hearts reoxygenated in the presence of L-NAME or with a combination of the antioxidants mercaptopropionylglycine and catalase. This protective effect during reoxygenation was abolished when excess L-arginine was added to the perfusate. These studies indicate that an acute and severe hypoxic insult is partly dependent on NO-mediated generation of the hydroxyl radical (43). The role of NO and reoxygenation in the chronically hypoxic heart subsequently subjected to an ischemic insult is unknown. The normal *in vivo* situation may represent a balance between the production of NO and oxygen-derived free radicals.

MODES OF CARDIOPROTECTION: ISCHEMIC PRECONDITIONING VERSUS CHRONIC HYPOXIA

In children with congenital heart disease, where aberrant anatomy prevents adequate access to all regions of the heart with cardioplegic solutions, this represents an increased risk factor for infants undergoing cardiac surgery (8, 17, 31). Protection of ischemic immature myocardium with cardioplegia is less than optimal despite its well-known benefits in adults, as shown by depletion of adenosine triphosphate (21, 42) and the generation of free radicals (12) and histologic injury (55). Thus, in pediatric cardiac surgery, additional cardioprotection by endogenous mechanisms such as preconditioning may be useful where conventional myocardial protection with cardioplegia is inadequate.

We demonstrated that hearts from infant rabbits normoxic from birth can be preconditioned by brief periods of ischemia. In contrast, hearts from infant rabbits hypoxic from birth were resistant to preconditioning, with the memory of preconditioning in immature normoxic hearts lost after 30 min following the preconditioning stimulus. The ATP-sensitive potassium (K_{ATP}) channel blocker 5-hydroxydecanoate completely abolished the cardioprotective effects of preconditioning in infant normoxic hearts, whereas 5-hydroxydecanoate did not affect recovery of postischemic function in nonpreconditioned normoxic hearts (5). We suggested that chronically hypoxic infant rabbit hearts are already protected and that additional cardioprotection by ischemic preconditioning is not possible. Thus, ischemic preconditioning may be able to serve a cardioprotective role in normoxic, but not chronically hypoxic, hearts. However, Ostadalova *et al.* (49) have suggested that this may not be present in the rat heart, where a combination of chronic hypoxia and ischemic preconditioning increased cardioprotection. In contrast, chronic hypoxia and ischemic

preconditioning, when applied separately, did not confer cardioprotection. Further studies are warranted to define the underlying mechanisms and to determine the optimal mode of cardioprotection in infant humans.

Cardioprotection of the myocardium can be induced by several ways, including the phase of ischemic preconditioning (7) and chronic hypoxia (3). The late phase of ischemic preconditioning refers to cardioprotection as manifest by decreased infarct size and increased recovery of postischemic developed pressure that becomes apparent 12–24 h after the initial ischemic preconditioning stimulus and lasts for 3–4 days (7). Similarities exist in the ability of these two diverse stimuli to protect the heart against subsequent ischemia. The magnitude of cardioprotection conferred by ischemic preconditioning is greater, whereas the duration of the cardioprotective effect lasts longer with chronic hypoxia (weeks) compared with ischemic preconditioning (days). Furthermore, both modes of cardioprotection possess a component of oxidative stress. However, distinct differences are present in the mechanisms underlying cardioprotection by ischemic preconditioning and adaptation to chronic hypoxia. In late preconditioning, NO generated from the inducible NOS (NOS2) isoform protects the heart against sustained ischemia (7). However, our studies with chronic hypoxia show that NO generated from the NOS3 isoform is responsible for protecting the heart against ischemia (56). Delayed preconditioning is mediated by activation of the mitochondrial K_{ATP} channel (15). Our studies show that both sarcolemmal and mitochondrial K_{ATP} channels mediate cardioprotection in chronically hypoxic hearts. Thus, the operative mechanisms by which adaptation to chronic hypoxia and late preconditioning protect the heart against ischemia are different.

REDOX-SENSITIVE CELL SIGNALING

During oxidative stress, cellular responses to ROS and RNS are critical to maintain cellular functions and in making the decision between cell survival and death. There are >100 genes identified that can be regulated by the cellular redox state. Redox-sensitive regulation of gene expression may be manifest at the transcriptional level, through alterations in mRNA stability, protein stability, and protein-protein interactions.

The effects of RNS may parallel the effects of ROS and antioxidants on cellular functions. NO biosynthesized from L-arginine has beneficial physiological effects, such as enhancing vasodilatation and inhibiting formation of platelet thrombi, and therefore is protective against cardiovascular disease (38). Indeed a common variant of NOS3 (NOS3 $Glu^{298} \rightarrow Asp$) is an important risk factor for coronary artery disease (26). Low levels of NO are produced constantly by the endothelium without the requirement for agonists such as acetylcholine, and this is achieved by normal shear stress induced by blood flow. The product of the Akt protooncogene (protein kinase B), a serine/threonine kinase, can directly phosphorylate NOS3 (16).

The mitochondria are a major cellular source of oxygen-derived free radicals and play a central role in energy metabolism. Ion channels within mitochondria also mediate myocardial injury during ischemia and reperfusion (48). Free radical intermediates of coenzyme Q in complex III, flavoproteins, and iron-sulfur proteins in complex I are significant sources of electron leakage during mitochondrial respiration. Generation of oxygen-derived free radicals during the initial phase of ischemic preconditioning has been proposed to trigger the activation of a signal transduction pathway that confers delayed protection against sustained ischemia (10, 11). Thus, oxygen-derived free radicals generated in mitochondria together with changes in redox equilibrium of mitochondrial electron carriers may serve as mediators of ischemia-induced myocardial injury.

Cardiomyocyte apoptosis mediated by ischemia and reperfusion is regulated by several redox-sensitive transcription factors and genes. Maulik *et al.* have shown that apoptosis occurs in conjunction with an increase in activator protein-1 (AP-1) and p53, coupled with a decrease in bcl-2 (44). Thus, mitochondrial electron carriers may serve as mediators of ischemia-induced myocardial injury and protection.

PROTEIN KINASES

ROS can act through several different pathways of signal transduction, making use of signaling molecules such as calcium, protein tyrosine kinases and protein tyrosine phosphatases, serine-threonine kinases, and phospholipases. Phosphorylation cascades are involved in many mechanisms for the transmission of extracellular signals from the plasma membrane to the cell nucleus. Numerous studies have found that oxidant treatment of cells produces elevations in protein phosphorylation and various protein kinase activities (1, 24, 25, 34, 46, 54).

Protein kinase C (PKC) family members are important mediators of hypoxia. In cardiomyocytes PKCα and PKCε translocate from soluble to particulate fractions of the cell in response to the stress of chronic hypoxia (18). The mitogen-activated protein kinases (MAPKs) are ubiquitous proteins activated by diverse stimuli and appear to mediate cellular responses, including proliferation, differentiation, and adaptation to stress (58). Three major MAPK families have been characterized, including the extracellular signal-regulated kinases (ERK or p42/44 MAPK), the c-Jun NH_2-terminal kinases (JNK), and the p38 MAPKs (58). ERKs are mainly involved in mediating anabolic processes such as cell division, growth, and differentiation, and the JNK and the p38 MAPKs are generally associated with cellular response to diverse stresses. Components of each of the MAPKs, or related pathway, contain redox-sensitive sites that provide the potential for modulation of the signaling via cellular redox status. ROS can activate MAPKs via a Ras-dependent mechanism (2, 13, 20, 51, 53). Oxidative stress has been shown to activate Ras (35). Evidence that NO reacts with cysteine-118, on the surface of Ras, to activate the G protein suggests that this residue may represent a redox-sensitive switch (35–37).

The clinical occurrence of protein kinases in adult humans was recently demonstrated by an increased activity of JNK and p38 MAPK in heart failure secondary to ischemic heart disease (9) and during cardiopulmonary bypass (59). The role of PKC and MAPKs in the mechanisms by which infant hearts adapt to chronic hypoxia and resist subsequent surgical ischemia has recently been investigated. Infant human and

rabbit hearts adapt to chronic hypoxia through activation of PKCε, p38 MAPK, and JNK, but not p42/44 MAPK. Phosphorylation and activation of heat shock protein 27 (Hsp27), a substrate for p38 MAPK, was present in chronically hypoxic, but not normoxic, hearts. Chronic hypoxia also caused phosphorylation of activating transcription factor-2 (ATF-2), a substrate for p38 MAPK. The possible relationship between chronic hypoxia and protein kinases is shown in Fig. 1. Activation of PKCε, p38 MAPK, and JNK, but not p42/44 MAPK, mediates cardioprotection in chronically hypoxic infant rabbits (52).

Ischemic preconditioning induces the translocation and activation of PKC. PKC appears to be the first element of a complex kinase cascade that is activated in preconditioned hearts. Current evidence indicates that PKC activates a tyrosine kinase that leads to the activation of p38 MAPK or JNK, or possibly both. The stimulation of these stress-activated protein kinases ultimately induces the opening of mitochondrial K_{ATP} channels.

NITRIC OXIDE SYNTHASE

NO plays a fundamental role in protecting the heart against ischemia/reperfusion injury. Biosynthesis of NO by constitutive NOS (endogenous NO) plays an important role in alleviating the severity of both reversible (myocardial stunning) and irreversible (myocardial infarction) damage incurred during ischemia/reperfusion in the heart (7). A similar protective effect can be produced by exogenous NO, given by means of NO-releasing agents or NOS substrates (27, 33). NO activates soluble guanylyl cyclase, which catalyzes the conversion of GTP to the signaling molecule cyclic GMP (6). Elevated cyclic GMP levels would be expected to be beneficial during myocardial ischemia, resulting in inhibition of Ca^{2+} influx into myocytes, decrease in myocardial contractility, reduction in myocardial oxygen consumption, and opening of sarcolemmal and mitochondrial K_{ATP} channels. The reduced Ca^{2+} current may alleviate the Ca^{2+} overload associated with acute myocardial ischemia, which is one of the major mechanisms of ischemic injury. Chronic hypoxia may represent a form of oxidative stress leading to the generation of ROS and RNS. This idea is supported by recent studies of Shi *et al.* (56), who reported that NOS3 protein is increased, but not its message, in chronically hypoxic infant hearts. Chronic hypoxia also decreases both the amount of caveolin-3 in the hearts and the amount that associates with NOS3. At the same time, increased NOS3 activity, increased cyclic GMP content, and nitrite and nitrate release occur in chronically hypoxic hearts. These findings suggest that one phenotypic change characterizing adaptation to chronic hypoxia is maximal biological activity of NO. These data show that chronic hypoxia increases NO generation from NOS3 to protect the myocardium against subsequent ischemia. Circulating NO levels, measured by nitrite and nitrate release from the heart, are increased in hearts from infant rabbits adapted to chronic hypoxia. These studies also suggest that chronic hypoxia represents a form of oxidative stress. In support of this idea, chronic hypoxia from birth also increases cardioprotection of isolated hearts by increasing the association of Hsp90 with NOS3. Normoxic infant hearts also generate more superoxide by a L-NAME-inhibitable mechanism than chronically hypoxic hearts. These critical protein–protein interactions help couple NOS3 activity to L-arginine metabolism and to limit superoxide anion generation from NOS3. Such changes in radical species generation by NOS3 increase NO production and help preserve NO activity in the heart,

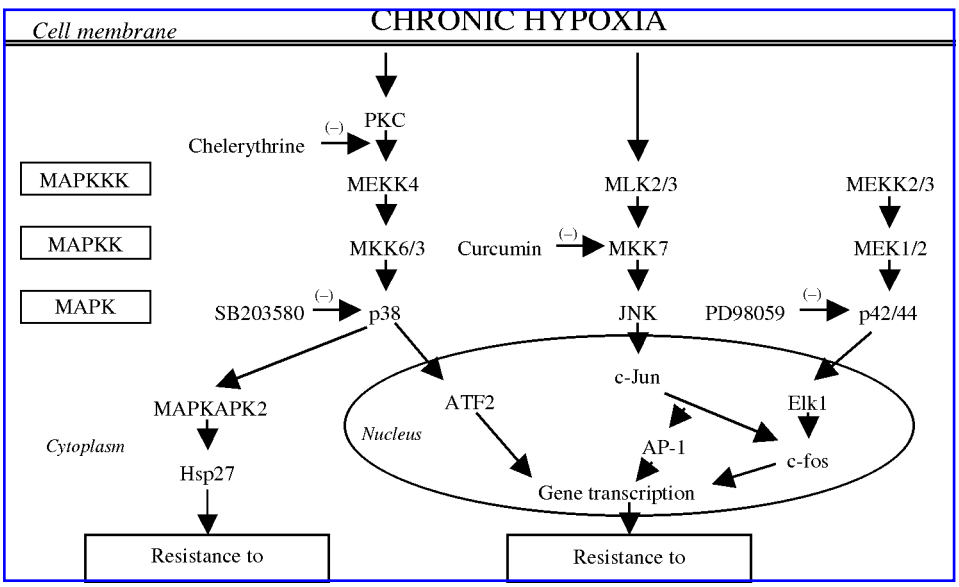

FIG. 1. Schematic representation of the impact of chronic hypoxia on protein kinase signaling pathways and their role in resistance to ischemia. Inhibitors of PKC (chelerythrine), p38 MAPK (SB203580), JNK (curcumin), but not p42/44 MAPK (PD98059), abolished the cardioprotective effects of chronic hypoxia (52). MAPKAPK2, MAPK-activated protein kinase 2; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase.

which increases resistance to ischemic reperfusion injury (57). Thus, NOS3 appears to critically important in adaptation of infant hearts to chronic hypoxia and in resistance to subsequent ischemia by regulating the production of ROS and RNS.

(K_{ATP}) CHANNELS

The K_{ATP} channel was first described in cardiac muscle cells in 1983 by Noma (47). Since this initial discovery, this channel has been found to be identical in almost all cell types. It is particularly important in the heart during metabolic impairment because of its regulation by the metabolic state of the cell. These channels are not active under normal physiological conditions, but are activated under pathophysiological conditions such as hypoxia and ischemia, resulting in a hyperpolarizing current flow and subsequent shortening of the cardiac action potential duration. Maintenance of a more negative membrane potential and shortening of action potential duration could result in a number of beneficial effects on the ischemic myocardium, such as a reduction in calcium influx via L-type calcium channels, the Na^+/Ca^{2+} exchanger, and the Na^+/H^+ exchanger. All of the above mechanisms will prevent calcium overload during ischemia and early reperfusion and result in a preservation of cellular energy stores and ultimately a reduction of myocyte and endothelial cell injury.

K_{ATP} channel openers have been shown to produce a beneficial effect on the myocardium in numerous models of ischemia (19), and the K_{ATP} channel has been demonstrated to be a key component of ischemic preconditioning (19). Initially, it was hypothesized that the surface or sarcolemmal K_{ATP} channel mediated protection observed after ischemic preconditioning; however, subsequent evidence suggested that the recently identified mitochondrial K_{ATP} channel may be the potassium channel mediating ischemic preconditioning-induced cardioprotection. However, we have demonstrated that both the sarcolemmal and mitochondrial K_{ATP} channels contribute to increased resistance of the chronically hypoxic heart to ischemia (32).

Opening of mitochondrial K_{ATP} channels reduces the inner mitochondrial membrane potential created by the proton pump and may lead to oxidation of the mitochondria. The mitochondrial redox state may be estimated by measuring fluorescence of FAD-linked enzyme in mitochondria. Using this technique, Liu *et al.* (41) showed that diazoxide reversibly oxidizes the mitochondrial matrix, based on specificity of the drug for the mitochondrial K_{ATP} channel. Thus, changes in redox state may exert profound effects on mitochondria mediating cardioprotection by ischemic preconditioning and adaptation to chronic hypoxia. The stress of increased production of ROS can trigger cardioprotection by activating protein kinases that may activate K_{ATP} channels. Opening of mitochondrial K_{ATP} channels with diazoxide and pinacidil increases ROS production (14), with the increase abolished by 5-hydroxydecanoate, a selective blocker of mitochondrial K_{ATP} channels (40).

Our laboratory was the first to show that the volatile anesthetic isoflurane induces delayed cardioprotection in infant rabbits at 24 h following exposure, which is manifest as an increase in recovery of postischemic developed pressure and a decrease in infarct size. This delayed cardioprotective effect of isoflurane is dose-dependent and mediated by the sarcolemmal and mitochondrial K_{ATP} channels. The cardioprotective effects of isoflurane appear indirect, as circulating levels of this volatile anesthetic are undetectable 24 h following exposure (60). Isoflurane inhibits complex I of the electron transport chain (23) and thus may trigger acute pharmacological preconditioning of the heart by the production of ROS. In support of this idea, isoflurane (45) and sevoflurane (29) acutely precondition myocardium against infarction by generation of ROS. This mechanism may also be responsible for triggering delayed cardioprotection with isoflurane.

ABBREVIATIONS

AP-1, activator protein-1; ATF-2, activating transcription factor-2; ERK, extracellular signal-regulated kinase; Hsp, heat shock protein; JNK, c-Jun NH_2-terminal kinase; K_{ATP} channel, ATP-dependent potassium channel; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; NOS3, endothelial isoform of nitric oxide synthase; PaO_2, partial pressure of oxygen in arterial blood; PKC, protein kinase C; RNS, reactive nitrogen species; ROS, reactive oxygen species.

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