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Mitochondrial contribution to the molecular mechanism of heart acclimatization to chronic hypoxia: role of nitric oxide

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1. ABSTRACT

A remarkable number of adaptive responses; including changes in the cardiovascular, respiratory and hematologic systems; takes place during acclimatization to natural or simulated high altitude. This adaptation to chronic hypoxia confers the heart an improved tolerance to all major deleterious consequences of acute O_2 deprivation, not only reducing infarct size but also alleviating post-ischemic contractile dysfunction and ventricular arrhythmias. There is growing evidence about the involvement of mitochondria and NO in the establishment of cardioprotection. This review focuses on evidence about the putative role of different effectors of heart acclimatization to chronic hypoxia. Along with classical parameters, we consider NO, specially that generated by mtNOS, mitochondrial respiratory chain, mitoK_{ATP} channels, reactive oxygen species and control of gene expression by HIF-1.

2. INTRODUCTION

During acclimatization to natural or simulated high altitude a remarkable number of adaptive responses takes place. As extensively described, respiratory, cardiovascular, and hematologic systems exhibit profound changes aimed to the maintenance of homeostasis. Adaptation to chronic hypoxia enhances cardiac tolerance to all major deleterious consequences of acute O_2 deprivation, not only reducing infarct size (1-3) but also alleviating post-ischemic contractile dysfunction (4-6) and ventricular arrhythmias (7,8). Myocardium resistance to O_2 deprivation is increased by systemic hypoxia, pharmacological intervention and ischemic preconditioning. The latter is the phenomenon whereby brief periods of ischemia enhance the tolerance of the heart to a subsequent ischemic stress. The cardioprotection conferred by chronic hypoxia lasts for several weeks; in contrast to preconditioning which may protect the

myocardium for several days (9). The relationship between long-term cardioprotection by acclimatization to chronic hypoxia and short-term protection by ischemic preconditioning is still unknown. The data about heart adaptation to chronic hypoxia is much less than the enormous quantity of publications dealing with the early and late phases of ischemic preconditioning. Thus, the complex mechanism underlying the increased tolerance of the chronically hypoxic heart to ischemic injury remains far less understood.

The ability of chronic hypoxia to protect myocardium was first described for individuals living at high altitude (10) and later confirmed by studies using simulated high altitude conditions in hypobaric chambers (1,11). Epidemiological observations on the protective effect of high altitude are consistent with the results of experimental studies using *in vitro* acute anoxia for testing the myocardial resistance of animals submitted to intermittent hypoxia (12). In addition, hypoxia from birth in immature rabbits increases the tolerance of isolated hearts to ischemia compared to age-matched normoxic rabbits (13). Interestingly, cardiac protection by adaptation to intermittent high altitude hypoxia may persist long after the regression of other hypoxia-induced adaptive changes, such as polycythemia, pulmonary hypertension and right ventricle hypertrophy (12).

Most of the factors proposed to be implicated in heart adaptation to hypoxia have been reported as part of the molecular mechanism of ischemic preconditioning. There is growing evidence about the involvement of mitochondria in the establishment of cardioprotection. The purpose of this assay is to review the evidence about the putative contribution of mitochondria and NO to the heart acclimatization to chronic hypoxia.

3. CLASSICAL PARAMETERS OF ACCLIMATIZATION TO CHRONIC HYPOXIA

Acclimatization to chronic hypoxia is characterized by a variety of functional changes that help to maintain homeostasis. The general pattern of physiological adaptation includes impairment in body weight gain (14,15), pulmonary hypertension (16), heart hypertrophy mainly associated to the compensatory response of the right ventricle to the functional overload caused by pulmonary hypertension, and polycythemia due to enhanced erythropoiesis (17).

Table 1 shows physiological parameters from rats submitted to hypobaric hypoxia (PO_2 = 53.8 kPa, 5000 m) for 1, 26 and 74 wk (15,18). Hematocrit values of hypoxic animals were increased since the first week of exposure; after 74 wk, they were 60% higher than the ones of normoxic rats. Rats submitted to simulated altitude for 1 week weighted 30% less than their control siblings. The difference was less marked at 26 wk (20%) and 74 wk (13%) of exposure. Right ventricle hypertrophy was evidenced after 10 wk of hypoxia, being almost 100% at 26 wk and later. On the other hand, exposure to hypoxia had no significant effect on left ventricle weight. Basal contractile parameters were modified in hypoxic animals. Developed tension (DT) declined during normoxic rats maturation but the decrease was less pronounced under chronic hypoxia, resulting in a significantly higher DT (64%) in hypoxic than in control rats at 26 wk of exposure (8 mo of age). At 18 mo of age (74 wk of exposure), however, hypoxic and normoxic animals exhibited similar DT values. The maximal rates of contraction and relaxation, +T and -T, followed a comparable pattern as DT; they were significantly higher in hypoxic 8-mo old rats (67% for +T and 52% for -T) than in their age matched controls. This preservation of the heart contractile function was associated to an increased mtNOS activity (see 4.5). Adaptation of adult rats to intermittent hypoxia for 5 weeks was also reported to increase right ventricle contractile function, determined as DT, +T and -T (19).

Table 2 shows different parameters of rat adaptation to natural high altitude chronic hypoxia in Cerro de Pasco, Peru (4340 m, PO_2 = 61.3 kPa), for up to 84 days. Hematocrit levels of rats exposed to high altitude were increased and were up to 40% higher than those of sea level rats after 84 days of exposure. On the contrary, body weight gain was decreased for animals exposed to high altitude: at 21 days of exposure it was 44% lower and at 84 days it was 35% lower than for sea level animals. Rats exposed to high altitude increased heart weight by about 60% (while control animals showed an increase of 24%) and also showed a right ventricle hypertrophy as compared with their sea level siblings, with values 128% higher than the ones for sea level rats after 84 days of exposure. At 84 days of life at sea level or at high altitude, right ventricle accounted for 17% or 30%, respectively, of total heart weight.

Such physiological adjustment may protect the heart under conditions that require enhanced work and consequently increased metabolism. Table 1 shows the DT values when tolerance to 60 min of hypoxia and recovery during a subsequent 30 min reoxygenation period was examined in the papillary muscles from rats submitted to hypobaric hypoxia or normoxia for 1 to 74 wk. Significant differences were found in animals exposed to hypoxia for 26 wk: force recovery during reoxygenation was significantly higher in hypoxic rats (71%) than in their controls (57%). During hypoxia and reoxygenation, +T and -T showed a behavior similar to DT: +T recovered 82% in adult hypoxic rats and 69% in controls, while -T recovery was 93% in adult hypoxic rats and 69% in their age-matched controls. No significant differences were observed between young rats (2 mo) after only one week of exposure to hypoxia, and in rats of old age (18 mo).

This effect of chronic hypoxia is well established and reproducible in models of intermittent or chronic hypobaric hypoxia showing that adaptation increases cardiac tolerance to all major deleterious consequences of acute O_2 deprivation (1-5,7,10). A model developed to reproduce cyanotic congenital heart disease, showed that adaptation to chronic normobaric hypoxia in infant rabbits confers resistance of the isolated neonatal heart to ischemia (6,13,20-23). Adaptation of rats to intermittent hypobaric

Table 1. Biological parameters of rats submitted to chronic hypoxia in a hypobaric chamber (PO_2=53.8 kPa, 5000 m) or to normoxia (PO_2=101.3 kPa, 0 m) for 1, 26 or 74 weeks

Parameter	Time of exposure (weeks)					
	1 N	1 H	26 N	26 H	74 N	74 H
[1]Hematocrit (%)	46.6±0.2	54.9±1.7	46.5±0.2	68.2±1.2	41.7±1.4	66.6±3.1
[1]Body weight (g)	288±22	205±12	658±3	530±34	680±19	595±77
[1]Right ventricle weight (g)	0.26±0.02	0.29±0.02	0.31±0.01	0.59±0.03	0.29±0.02	0.56±0.02
[1]Left ventricle weight (g)	0.68±0.05	0.59±0.03	1.07±0.04	1.15±0.06	1.62±0.07	1.29±0.02
[1]Developed tension ($g.mm^{-2}$)	3.6±0.3	3.7±0.2	1.7±0.1	2.8±0.1	1.8±0.3	1.8±0.2
[1]Developed tension H/R ($g.mm^{-2}$)	2.0±0.3	2.3±0.3	1.0±0.1	2.0±0.1	0.8±0.1	1.5±0.3
[2]mtNOS activity (nmol $NO.min^{-1}.mg\ protein^{-1}$)	0.68±0.03	0.70±0.02	0.64±0.02	0.88±0.02	0.55±0.02	0.95±0.03
[2]NADH-cytochrome c activity (nmol $NADH.min^{-1}.mg\ protein^{-1}$)	7.1±0.4	7.0±0.4	6.2±0.3	5.8±0.3	4.6±0.3	4.2±0.2
[2]Cytochrome oxidase activity ($k'.mg\ protein^{-1}$)	8.70±0.20	8.50±0.14	8.06±0.15	7.85±0.12	7.76±0.12	7.53±0.20
[2]Cytochrome aa_3 content ($nmol.mg\ protein^{-1}$)	0.11±0.01	0.10±0.01	0.15±0.01	0.15±0.02	0.14±0.02	0.13±0.02
[2]Cytochrome c content ($nmol.mg\ protein^{-1}$)	0.19±0.01	0.20±0.01	0.27±0.02	0.27±0.01	0.26±0.01	0.26±0.02

Adapted with permission from [1](15) and [2](18). Chronic hypoxia was experimentally developed in Wistar rats (7 wk old), placed into a hypobaric chamber (H) for 1 wk (2-mo-old,), 26 wk (8-mo-old), 74 wk (18-mo-old) or kept in normoxia (N). H/R: hypoxia-reoxigenation.

Table 2. Heart acclimatization to life at high altitude at Cerro de Pasco, Peru (4340 m, PO_2=61.3 kPa) for 21 or 84 days

	Time of exposure (days)			
Parameters	**[1]SL-21**	**[1]HA-21**	**SL-84**	**HA-84**
Hematocrit (%)	47±2.7	60.7±0.8	50.0±1.1	68.5±3.4
Body weight gain (g)	44.5±5.8	25.0±8.5	90.6±10.0	59.1±9.0
Heart weight (g)	1.08±0.07	1.23±0.02	1.22±0.07	1.59±0.36
Right ventricle weight (g)	0.22±0.03	0.25±0.06	0.21±0.03	0.48±0.08
Left ventricle weight (g)	0.81±0.14	0.80±0.04	0.90±0.10	0.74±0.06
mtNOS activity ($nmol\ NO.min^{-1}.mg\ protein^{-1}$)	0.81±0.04	1.04±0.03	0.74±0.08	1.26±0.09
mtNOS expression (%)	nd	nd	100	159±10
NADH-cytochrome c activity ($nmol\ NADH.min^{-1}.mg\ protein^{-1}$)	127±4	158±5	124±5	160±8
Succinate-cytochrome c activity ($nmol.min^{-1}.mg\ protein^{-1}$)	16.6±1.6	14.8±1.3	16.3±1.9	18.7±2.3
Cytochrome oxidase activity ($k'.mg\ protein^{-1}$)	19.0±1.3	21.2±1.9	18.2±1.6	21.5±1.9
Cytochrome aa_3 content ($nmol.mg\ protein^{-1}$)	nd	nd	0.58±0.02	0.68±0.03
Cytochrome c content ($nmol.mg\ protein^{-1}$)	nd	nd	0.68±0.02	0.90±0.04

[1]:Adapted with permission from (14). SL: Sea level, HA: High altitude, nd: not determined

hypoxia (53.8 kPa) protects the heart against ischemia-induced arrhythmias (8,12). More recently, short episodes of intermittent or continuous hypoxia were shown to induce delayed cardioprotection (24,25). It is well recognized that chronic exposure to hypoxia results in improved heart tolerance to ischemia reperfusion injury.

4. NITRIC OXIDE

4.1. Role of NO in cardiac function

Nitric oxide (NO) has a critical function as an endogenous modulator of vascular tone (26,27). Moreover, heart NO, regulated by the Ca^{2+} levels of the contraction and relaxation cycles and produced by cytosolic and mitochondrial NO synthases (NOS), is essential for heart homeostasis and mechanical activity (28,29). Nitric oxide activates soluble guanylate cyclase by binding to its heme group. This activation leads to the production of 3',5'-cyclic guanosine monophosphate (cGMP), which in turn activates protein kinase G and a cascade of biological signaling events (30). It is clear that cGMP plays a role in cardiac function and ion channel regulation (29) and that it is involved in eliciting NO effects on myocardial excitation-contraction coupling (29). Furthermore, NO acts in signaling and regulation of respiration through cGMP-independent ways (31). Nitric oxide has been identified as a high affinity and reversible inhibitor of cytochrome oxidase activity (32,33); at submicromolar concentrations it competes with O_2 for the binding site at the binuclear center formed by cytochrome a_3^{2+} and Cu_B^{+} of cytochrome oxidase. In addition to binding to the heme iron in proteins such as guanylate cyclase and cytochrome oxidase, NO can modify proteins by nitrosylation (34). Ion channel of most classes such as plasmalemmal L-type Ca^{2+} channels and the sarcoplasmic reticulum ryanodine receptor are targets for S-nitrosylation within the heart (35). The ubiquitous effects exerted by NO in cells are conveyed in large part by this mechanism of protein regulation (36,37).

Nitric oxide has opposite effects on cardiac contractility, depending the cellular compartment in which it is generated and the rate at which it is generated (see 4.3 and 4.5). This specificity is conferred through spatial localization of NOSs to signaling modules (38). There is a growing consensus that NO modulates the activity of several key Ca^{2+} channel involved in excitation-contraction coupling as well as mitochondrial respiratory complexes. To accomplish this regulation, different NOS isoforms are spatially confined in distinct cellular microdomains involved in excitation-contraction coupling (29).

Nitric oxide influences myocardial contractility, ventricular relaxation and mitochondrial respiration through the distinct mechanisms described above and its concentrations fluctuate with the cardiac cycle, in the submicromolar range, peaking in late diastole (28). Indeed,

NO may have both positive and negative influences on cardiac contraction that are both physiologically relevant to excitation-contraction coupling (29). The involvement of NO in the inhibition of respiration and in intra or intercellular signaling seems to afford its central role in the molecular mechanism of cardioprotection.

4.2. NO is an effector of cardioprotection

As detailed previously, NO exerts a number of actions that would be expected to be beneficial during myocardial ischemia-reperfusion (39). Regardless the model used, NO has been consistently implicated as a required factor in the cardioprotective actions of various interventions (40). The administration of NO donor S-nitrosoglutathione (GSNO) during a 35-min ischemic arrest dramatically improves functional recovery after ischemia (41). A similar effect is achieved after addition of NOS substrates (42). In contrast, treatment with NOS inhibitors such as N^{ω}-nitro-methyl-esther (L-NAME) results in an exacerbation of heart contractile dysfunction after ischemia-reperfusion (43).

Nitric oxide has been extensively proposed as one of the molecular messengers involved in the cardiac protection but reports about the effect of chronic hypoxia on NO synthesis and the function of NO in that mechanism of cardioprotection are not decisive (12,14,18). Pretreatment of chronically hypoxic hearts with GSNO before global ischemia-reperfusion did not confer further contractility recovery in hypoxic hearts, but it did improve function recovery in normoxic hearts. This indicates that in hypoxic hearts, endogenous NO production is already enhanced and the molecular mechanism triggered by NO is already activated. On the contrary, NOS inhibitors completely abolished cardioprotective effect of chronic hypoxia (21) but had no effect on the recovery of function in normoxic hearts. Subjects submitted to natural chronic hypoxia, *i.e.* high altitude, have increased levels of NO stable catabolites in blood (44). Therefore, the adaptive mechanisms of the cardiovascular system seem to trigger the activation of NO synthesis in specific cells and tissues which are involved in the adaptation to high altitude.

4.3. NOS isoforms implicated in cardioprotection

The source of NO involved in cardioprotection is another issue that remains under discussion. Nitric oxide biosynthesis is enhanced during early and late ischemic preconditioning (39). It has been proposed that two different NOS isoforms are sequentially involved in late preconditioning, with eNOS generating the NO that initiates the development of the early response and iNOS then producing the NO that protects heart against recurrent ischemia on late preconditioning (45). Late preconditioning is associated to an increase in the protein expression (47%) and activity (227%) of iNOS, but not of eNOS or nNOS. The selective up-regulation of iNOS that takes places during late ischemic preconditioning was evidenced using a model of mice hearts lacking iNOS gene (46). Disruption of iNOS gene had no effect on early preconditioning or on infarct size in the absence of preconditioning but it totally abrogated the infarct-sparing effect of late preconditioning. Myocardial ischemia reperfusion injury was exacerbated in absence of eNOS gene (47). In addition, the use of a line of transgenic mice showed that eNOS overexpression significantly attenuates myocardial reperfusion injury but fails to protect myocardium against post-ischemic contractile dysfunction (48). Another group used an adenoviral vector encoding human iNOS and reported that myocardial overexpression of iNOS afforded powerful cardioprotection against ischemia-reperfusion the magnitude of which is equivalent to that of late preconditioning (49). On the contrary, the absence of iNOS gene actually exacerbates multiple parameters of cardiac injury in mice (50).

Concerning chronic hypoxia models, the NOS isoform implicated in conferring increased tolerance to lethal ischemic injury still remains to be established. Different groups have reported iNOS up-regulation during rat exposition to chronic hypoxia (12). Exposure of rats for 8, 15 and 21 days increased iNOS abundance by about 120-200% in right ventricle and by 90-110% in left ventricle (51). In the same study, NOS activity was up-regulated by hypoxia showing a maximum in right ventricle at 15 days of exposure of 160% above normoxic controls. Treatment with L-NAME caused a decrease in right ventricle hypertrophy in hypoxia. A similar model was used to show that iNOS protein levels, assessed by Western blotting and inmunohistochemical techniques, were increased by 4 and 8 fold respectively during a 14-days exposure to hypoxia (52). This data was supported by the augmented iNOS mRNA levels of hypoxic rats. In addition, the authors evaluated NOS activity which resulted 5 times higher in hypoxic rats.

In contrast, some reports indicate that eNOS is the isoform up-regulated during heart acclimatization to chronic hypoxia. Adaptation of rabbit hearts to chronic hypoxia from birth increased eNOS mRNA levels (increased expression by 238%), constitutive NOS activity (3.3 times higher) as well as the release of nitrite, nitrate (2 to 3 times greater) and cGMP and tissue cGMP content (21). These findings were associated with increased tolerance to ischemia. Further studies indicated that chronic hypoxia decreases the amount of caveolin-3 in heart homogenates by about 2 fold and that this fact could be involved in the mechanism by which chronic hypoxia increases eNOS activity in hypoxic rabbit hearts (53). In addition, it was reported that chronic hypoxia increased eNOS expression in rat heart left (55%) and right (35%) ventricles (54). Indeed, the persistence of cardioprotection conferred by adaptation to hypoxia from birth on subsequent exposure to normoxia is associated with enhanced NOS activity and activation of K_{ATP} channels (55).

Undoubtedly, adaptation to chronic hypoxia enhances heart NOS expression and activity. Nevertheless, the subcellular localization and the identity of the implicated NOS isoform is still a subject that needs a careful revision.

4.4. Mitochondria and nitric oxide

Mitochondria are organelles that, apart from being the cellular powerhouses, play a pivotal role in the

control of cell survival (56). Emerging evidence points to mitochondria as essential players in NO-dependent cardioprotection (40) as their normal function is tightly modulated by NO steady state levels (57). Nitric oxide exerts several distinct effects in mitochondria: it competitively inhibits cytochrome oxidase (32,33,57-59); it regulates the rate of production of species derived from the partial reduction of O_2 such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (59); it activates mitoK_{ATP} channels (60); it prevents permeability transition pore (MPT) formation in a concentration dependent manner (61). Nitric oxide is indeed the first molecule that fulfills the requirement for a physiological modulator of respiration on the basis of its O_2 competitive inhibition of cytochrome oxidase activity (32,58); it is produced in the tissues at a fair rate high enough to exhibit an inhibitory effect on cytochrome oxidase that extends the O_2 gradient into the tissues (59, 62). Furthermore, NO was found to trigger mitochondrial biogenesis in several cell types and tissues including heart (63). This wide spectrum of actions supports the idea of the critical role of the interaction of NO and mitochondria in the onset of cardioprotection.

Moreover, almost a decade ago, the existence of a focused NO production by mitochondrial NOS (mtNOS) was described (64,65). These reports prompted the concept that mtNOS may serve to adjust mitochondrial function to cell energy demands and established this organelle as a source of signaling molecules such as H_2O_2 and NO itself (66-68). Nitric oxide synthase activity has been measured in mouse cardiomyocyte mitochondria upon Ca^{2+} addition to the reaction medium using a porphyrinic NO microsensor (69). The presence of a mtNOS in heart, where NO plays a role in contractility, has been confirmed by a series of reports (70-73).

Since this enzyme was described, its relevance in mitochondrial bioenergetics and physiology has been under consideration. The biological function of mtNOS is currently discussed in terms of inhibition of tissue respiration (58,74), modulation of tissue O_2 gradients (59,62), induction of apoptosis (75), and nitrative and nitrosative stress in inflammation (73). Physiological roles for mtNOS have been postulated in cell signaling, mitochondrial pathology, aging, dystrophin deficiency, inflammation and cancer (76). Mitochondrial NOS activity has been found up-regulated in cold acclimation (77). Treatment with enalapril, the inhibitor of the angiotensin converting enzyme, for 14-28 days produced an increase of 87% in production of NO by heart mitochondrial membranes (70,71). Accordingly, there was an enhancement in mtNOS functional activity, evidenced both through a decrease in O_2 consumption (1.5-2.6 times) and an increase in H_2O_2 production (1.9-3.5 times) (78). Heart mtNOS activity was found modulated by mitochondrial membrane potential (79). Mitochondrial NO production decreased by about 50% in transition from state 4 to state 3. Thus, heart mtNOS is regulated by the mitochondrial metabolic state. In addition, heart mtNOS activity was reported to be up-regulated in experimental endotoxemia after LPS treatment (73). Therefore, this enzyme responds to a variety of physiological, pathological and pharmacological agents.

4.5. Role of mtNOS in acclimatization to chronic hypoxia

Liver mtNOS activity was reported increased by 67% after acute exposition (25 min, 6 h before mitochondrial isolation) to 8% (40 kPa) O_2 (80). However, in that study, mtNOS expression was assessed by Western blotting and showed bands of similar intensity compared with the normoxic controls. In suspensions of isolated rat liver mitochondria, hypoxia causes an increase in NO concentration (81). Our data extend this view suggesting that mtNOS-derived-NO may be the most important source of cardiomyocyte NO in normoxia and chronic hypoxia. Heart mtNOS activity was significantly higher (20-60%) in rats submitted to chronic hypoxia for up to 74 wk than in their control siblings (Table 1). The effect of hypobaric hypoxia was marked enough to counteract the age-associated decrease of mtNOS activity and to reach a higher activity that the one corresponding to young animals (2 mo old) (18,72). This up-regulation in hypoxia was associated to a preservation of papillary muscle contractile parameters upon aging (see 3). At 8 mo of age (26 wk of exposure), the protective effect was maximal: mtNOS activity and DT from hypoxic hearts were 35% and 64% higher than the ones from normoxic controls. At 18 mo of age (74 wk of exposure), hypoxic rats showed a 60% increase in mtNOS activity but no significant difference in DT when compared with their age-matched controls. Papillary muscle +T and -T had a similar pattern to the one of developed tension (15).

In that study, an increased left ventricle biochemical mtNOS activity was found also associated with improved recovery after an *in vitro* sequence of 60 min hypoxia-30 min reoxygenation in heart. Hypoxia was related to increased DT and mtNOS activity values at 8 mo of age: hypoxic rats showed an increase of 35% in mtNOS activity associated with a DT 100% higher than their age matched controls. +T and -T followed a similar pattern (15).

Exposition to high altitude (4340 m, 61.3 kPa, Cerro de Pasco, Peru) for up to 84 days significantly enhanced heart mtNOS activity by 70% (Table 2). The NOS protein expression was also increased, by 60%, after 84 days of life at high altitude. Animals living at sea level showed no significant changes in heart mtNOS activity. Nitric oxide synthase activity measured in post mitochondrial supernatant was not affected. Heart mtNOS showed a good correlation with hematocrit levels strongly supporting the idea of a common regulation at transcriptional level for erythropoietin and NOS protein expression (see 7).

The fraction of the total NO cellular production generated by the different NOS isoforms is a subject starting to be understood. The contribution of heart mtNOS to cardiomyocyte NO production is high enough (90%) to sustain a relevant role of this enzyme in cardioprotection (82). These data clearly indicate that mtNOS and mitochondria are the main source of cellular NO in cardiomyocytes. The increase in mitochondrial NO (18), by modulating the activity of the respiratory chain, would

allow O_2 to diffuse further and to reach more mitochondria (57) providing a way of increasing O_2 extraction, which could help make the heart more efficient. Moreover, the rate of mitochondrial H_2O_2 production was reported as directly regulated by mtNOS activity (78). At present, not only mitochondrial H_2O_2 (66, 67) but also mitochondrial NO (63, 67, 68) are considered together as a pleitropic signal that is able to regulate the expression of genes involved in the control of cell metabolism and proliferation. In view of these evidences, increased heart NO production and NO steady state levels seem essential for the development of cardioprotection. Increased mtNOS activity contributes to signaling and improved contractility described after exposure to chronic hypoxia.

5. MITOCHONDRIAL FUNCTION IN CHRONIC HYPOXIA

During chronic exposure to hypoxia there is an increased requirement for energy in conditions of low O_2 availability. Thereby, mitochondrial function may be changed to satisfy the energy demands of the cell. A normal O_2 delivery to the heart tissue is approached in chronic mild hypoxia by an increase in mitochondrial number more homogenously distributed and a decrease in mitochondrial size (83, 84). These features could be linked to an enlarged surface-to-volume ratio and therefore would be an effective mechanism of adaptation to low O_2 pressure in the heart. Cells with clustered mitochondria require higher O_2 concentrations to maintain functions than cells with uniformly distributed mitochondria (85). It follows that distribution of mitochondria alters the sensitivity of the tissues to hypoxia.

5.1. Mitochondrial respiratory chain

During adaptation to chronic hypoxia no differences were found in the intrinsic properties of mitochondria, *i.e.* maximal physiological rate of respiration (state 3, active respiration), resting respiration (state 4), ADP/O ratio and respiratory control (83). The activities of NADH-cytochrome *c* reductase and cytochrome oxidase were not affected by hypobaric hypoxia but exhibited a decline of about 36% and 12%, respectively, with aging (Table 1) (18). The contents of cytochromes aa_3 and *c* were similar in the left ventricles of hypoxic and normoxic animals. The activities of the respiratory chain electron transfer complexes of heart mitochondria were also measured to assess mitochondrial function in rats exposed to high altitude (14). Rats exposed for up to 84 days to high altitude showed a NADH-cytochrome *c* reductase (Complexes I-III) activity slightly (15%) higher than that of rats kept at sea level (Table 2). By the contrary, succinate-cytochrome *c* reductase (Complexes II-III) and cytochrome oxidase (Complex IV) activities were unaffected by exposition to high altitude. In agreement, cytochrome aa_3 and *c* contents were similar in heart mitochondria of animals exposed to sea level and high altitude.

In a study designed to assess the differential energy metabolism in each ventricle of the heart during the acclimatization, it was shown that the oxidative capacity of the overloaded right ventricle is not altered during adaptation. Maximal reaction velocity of cytochrome oxidase was about the same in the two ventricles, and there were no significant differences between control and hypoxia-adapted animals (86). It has been shown that chronic hypoxia induces a reduction in the efficacy of the coupling of the mitochondrial creatine kinase activity to mitochondrial oxidative phosphorylation. This can lead to a decrease in the effect of ADP on mitochondrial respiration and, possibly, to a reorganization of the cellular systems implicated in energy transfer (87).

Interestingly, mitochondrial electron transport chain is involved in development of cardioprotection as potential regulator of the stabilization of the transcription factor HIF-1alpha (see 7) (88). The fact that cells lacking subunits of complexes I, III and IV prevent hypoxic stabilization of this transcription factor was the major initial evidence (89). Moreover, a variety of respiratory chain inhibitors have been shown to exhibit the same effect (90,91). Studies using knockdown approaches provide further evidence about the role of mitochondrial electron transport chain involvement in hypoxic stabilization of HIF-1 alpha (92-94). The pathway by which mitochondrial respiratory chain function is linked to HIF-1 alpha stabilization most likely includes reactive oxygen species (*i.e.* O_2^- and H_2O_2) generation.

5.2. Superoxide anion and hydrogen peroxide production

Reactive oxygen species are generated by incomplete reduction of O_2 within mitochondrial electron transport chain in a process that is regulated by the metabolic state (95). The enhanced mitochondrial generation of these species appears to be an event implicated in the onset of cardioprotection (88). Indeed, changes in H_2O_2 steady state concentrations play an important second messenger role in a variety of signaling pathways that are critical for cell physiology (66,96).

In the absence of adaptation to chronic hypoxia or ischemic preconditioning, exposure to low concentrations of O_2^- can reproduce cardioprotection on infarct size and postischemic recovery of left ventricular function in a process prevented by the administration of superoxide dismutase and catalase (97). One postulated mechanism by which O_2^- and H_2O_2 may be distinguished as prominent mediators of adaptation to hypoxia involves the stabilization of transcription factor HIF-1alpha (88). Hypoxia increases mitochondrial free radical production (91,94). Indeed, overexpression of antioxidant enzymes such as catalase or glutathione peroxidase that scavenge H_2O_2 prevent hypoxic stabilization of HIF-1alpha, indicating that this species is the major intracellular signaling molecule required.

It has been proposed that mitoK_{ATP} channel opening triggers cardioprotection by causing a moderate increase in the generation of reactive oxygen species, which serve as second messengers to activate kinases within the cardioprotective signaling pathway (see 6) (98-101). The pharmacological opening of mitoK_{ATP} channels generates free radicals that trigger entrance into a state

similar to that of preconditioned heart and activation of kinases (102). In another study, the direct detection of ROS production in isolated rat ventricle myocytes was used to show that mitoK$_{ATP}$ channels opening increases reactive oxygen species production (101). The use of antioxidants or of a mitoK$_{ATP}$ channel blocker produced a decrease in reactive oxygen species, this latter seem to afford the link between mitoK$_{ATP}$ channels opening and kinase activation (103). Even if none of these studies focused on specifying the identity of the involved species, O_2^- and H_2O_2 would be the reasonable candidates for the execution of these functions.

Interestingly and as mentioned before, NO has been reported to inhibit electron transfer at complex III (ubiquinol-cytochrome c reductase) and to increase the mitochondrial rate of O_2^- and H_2O_2 generation (59). Moreover, the rate of mitochondrial H_2O_2 production was reported as directly regulated by mtNOS activity (78). Thus, NO produced by mtNOS may be acting also at this level of the cascade of events implicated in the building of enhanced cardiac tolerance to ischemia-reperfusion deleterious effects.

6. MITOCHONDRIAL ATP-SENSITIVE K^+ CHANNEL

There is strong evidence implicating K$_{ATP}$ channels, particularly those located in the inner mitochondrial membrane (mitoK$_{ATP}$ channels) in the protective mechanism afforded by adaptation to chronic hypoxia (104-106). Mitochondrial K$_{ATP}$ channels are found in the inner mitochondrial membrane where they are involved in different functions (107-109). The utilization of mitoK$_{ATP}$ channel openers and blockers in different models supported the hypothesis that mitoK$_{ATP}$ is an important mediator of heart cellular protection against ischemia reperfusion injury. Diazoxide, a specific mitoK$_{ATP}$ channel opener, protects the heart against various manifestations of ischemia-reperfusion injury and its effects are abrogated by a selective mitoK$_{ATP}$ channel blocker as 5-hydroxydecanoate (5-HD) (99,102,104,105,110-112).

Increased tolerance to ischemia exhibited by chronically hypoxia hearts is associated to increased activation of K$_{ATP}$ channels. Whereas 5-HD eliminated the protective effect of chronic hypoxia on the reduction of infarct size and post-ischemic recovery of contractile function; it had no effect in normoxic hearts. On the contrary, diazoxide significantly increased the recovery of contractile function, reduced infarct size and the number of ischemic ventricular arrhythmias in normoxic hearts to the same extent achieved by chronic hypoxia, but had no effect in the hypoxic hearts (8, 113). Furthermore, the use of a novel inhibitor of mitoK$_{ATP}$ channel and activator of sarcolemmal K$_{ATP}$ channel, MCC-134, abolished the protective effect of chronic hypoxia but had no effect when administered to normoxic animals (114). The model of adaptation of rabbit hearts to normobaric hypoxia from birth was also used to study the involvement of K$_{ATP}$ channels in the mechanism of cardioprotection. (6,20,22).

As the existing evidence strongly suggests that mitoK$_{ATP}$ are part of the cardioprotective mechanism of chronic hypoxia, the quantitative importance of mitoK$_{ATP}$ is starting to be understood. Several consequences of mitoK$_{ATP}$ channel activation are likely to improve mitochondrial function after ischemia. Mitochondrial K$_{ATP}$ opening results in K^+ influx into mitochondria, expansion of matrix volume (mitochondrial swelling), and a dissipation of membrane potential established by the proton pump, slowed ATP production, release of accumulated Ca^{2+} ions and ROS production (105,115,116). Acute activation of the mitoK$_{ATP}$ channel results in a reduction in the driving force for ATP synthesis. MitoK$_{ATP}$ channels seem to be tonically active in hypoxic hearts and may act in the modulation of mitochondrial bioenergetics, which renders the hypoxic heart more resistant to myocardial ischemia by permitting efficient energy transformation during reperfusion (22,111).

Moreover, mitoK$_{ATP}$ channels are starting to be linked with other components implicated in the heart adaptation to chronic hypoxia. Nitric oxide is capable of activating mitoK$_{ATP}$ channel (60) and is postulated to be one of the signals in mitoK$_{ATP}$ channel activation via soluble guanylyl cyclase, causing cGMP accumulation and cGMP dependent protein kinase activation (117,118). In addition, mitoK$_{ATP}$ channel function can be regulated by protein kinase C, which is permanently activated by chronic hypoxia (51,119,120). It was postulated that H_2O_2 triggers preconditioning by causing activation of the mitoK$_{ATP}$ channel, which then induces generation of H_2O_2 and of NO that are required for preconditioning protection (121).

The data presented is in line with results reported by groups working with ischemic preconditioning models, in which, an increasing body of evidence based mostly on pharmacological approaches, indicates that mitoK$_{ATP}$ channels are the more likely effectors of protection (60,110,112,116,122-124). It appears that mitoK$_{ATP}$ channels constitute a central component of both short and long-term cardioprotective mechanisms.

7. HYPOXIA-INDUCIBLE TRANSCRIPTION FACTOR 1

Cells are able to sense low O_2 concentrations through a hypoxic response pathway that results in changes in gene expression. Hypoxia-inducible transcription factor 1 (HIF-1) is expressed in all cell types and functions as a master regulator of O_2 homeostasis because it plays a critical role in the response to hypoxia by regulating the expression of over 70 target genes at the transcriptional level (125). Genes up-regulated by HIF-1 are involved in physiological processes such as erythropoiesis, angiogenesis, and glycolysis; that are critical to maintain O_2 homeostasis (126). These are examples of systemic, local tissue, and intracellular adaptive responses to hypoxia, respectively. HIFs are composed of two subunits: the hypoxia-regulated alpha subunit and the O_2-insensitive HIF-1beta ¸ subunit. Under normoxic conditions, HIF-1 alpha is continuously degraded. In hypoxia, it accumulates instantaneously within the nucleus where it binds to HIF-1 beta to recognize HIF-responsive elements (HREs) among

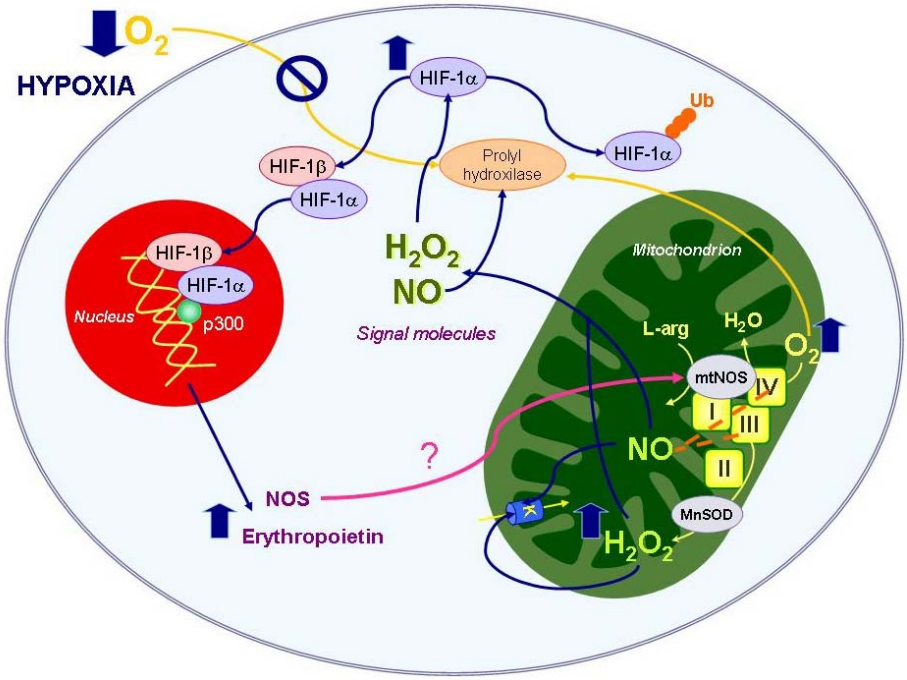

Figure 1. Schematic representation of the proposed relationships among the effectors of cardioprotection conferred by chronic hypoxia. Solid line narrows represent activation and dashed lines represent inhibition.

the promoters of hypoxia-responsive target genes (125). The O_2-dependent degradation of HIF-1 alpha is initiated by hydroxylation. A family of prolyl hydroxylases (127,128) that uses O_2 as substrate to catalyze hydroxylation of proline residues of HIF-1 alpha in a polypeptide segment known as the O_2-dependent degradation domain (129), is responsible for this crucial regulatory step. The Km for O_2 of the prolyl hydroxylases is very high (130). That means that O_2 concentration is rate limiting in the hydroxylation by these enzymes. The hydroxylation of HIF-1 alpha triggers its degradation via ubiquitination and degradation by the 26S proteasome (131,132). As a consequence of its tightly regulated degradation, HIF-1 alpha has a very short half-life (133), which makes it suitable to sense very effectively the cellular O_2 concentration. As mentioned before, there are additional levels of HIF modulation (127): the secondary consequences of changes in O_2 concentration, such as alteration in intracellular redox potentials or the amount of ROS, may influence HIF induction (134). Nitric oxide prevents the stabilization of HIF-1 alpha as a result of an increase in prolyl hydroxylase-dependent degradation of HIF-1 alpha (135).

HIF-1, in turn, is essential for the hypoxic regulation of iNOS gene expression. The sequences around HIF-1 site present in the 5´-flanking region of the iNOS gene and the 3´-enhancer of the erythropoietin gene show a region of similarity that suggests a similar regulation pattern for these proteins (136). It binds to identified HREs in the promoter region of iNOS gene (137). Interestingly, activation of HIF-1 in normoxic myocardium via a gene specific non pharmacological approach produces NO-dependent cardioprotection; a phenomenon not observed if the mice used are iNOS-/- (138). In a model of rat exposed to chronic hypoxia, HIF-1 alpha mRNA levels were found differentially increased in heart left (100%) and right (50%) ventricles (54). It is clear that there is an interplay between NO and H_2O_2 in the regulation of HIF-1alpha activity.

8. CONCLUSIONS AND PERSPECTIVES

When tissues are challenged by hypoxia, the expression of a number of physiologically important proteins such as erythropoietin and nitric oxide synthases is increased. Physiological changes involves changes in the pattern of gene expression. The establishment of long term-cardioprotection constitutes the hallmark of heart adaptation to low O_2 pressures. We have reviewed the current views on the mechanisms responsible for enhanced myocardium tolerance to hypoxia and their relationship with mitochondria (Figure 1). It is evident that all the actors presented here interact in a not yet fully understood pathway leading to cardioprotection. Increased NO production in chronically hypoxic hearts is part of an adaptive response in which several signaling pathways are involved and NO is clearly a common messenger at different stages of the mechanism. Nevertheless, the subcellular localization and the identity of the implicated NOS isoform is a subject that needs a careful revision. Mainly because the studies performed do not differentiate subcellular compartments. The distribution of NOS isoforms in the different compartments, and the NO diffusion between mitochondria and cytosol underlie the role of NO in the regulation of cellular homeostasis and in biochemical and genetic signaling. As more evidence about

the crucial role of mitochondria in the molecular mechanism of cardioprotection becomes available, it is likely that the understanding of the relevance of mitochondrially-generated NO will be achieved.

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