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Review

Perceived hyperoxia: Oxygen-induced remodeling of the reoxygenated heart

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Abstract

Focal coronary artery blockage followed by further reperfusion injury is commonly involved in myocardial infarction. The injured heart has some inherent reparative responses. Although such natural healing mechanisms seem to be inefficient, a clear understanding of the underlying principles of myocardial healing holds the key to successful therapy. Under normoxic conditions, pO_2 ranges from 90 to <3 Torr in mammalian organs with the heart at ~35 Torr (5%) and arterial blood at ~100 Torr. Thus, "normoxia" for cells is an adjustable variable. In response to chronic moderate hypoxia, cells lower their normoxia set-point such that reoxygenation-dependent relative elevation of pO_2 ($+\Delta pO_2$) results in perceived hyperoxia. Perceived hyperoxia induces differentiation of cardiac fibroblasts to myofibroblasts in the peri-infarct region and represents a significant factor supporting myocardial healing. The oxygen-sensitive signaling pathways involved have been characterized and point towards a central role of p21, TGFβ and p38MAPK. That low oxygen ambience serves as a cue to trigger angiogenesis is a well-accepted notion. Studies related to perceived hyperoxia establish that the sensing of oxygen environment is not limited to hypoxia. It demonstrates that in addition to being a trigger for injury as is widely recognized, reoxygenation insult has a built-in component of tissue remodeling in the peri-infarct region induced by perceived hyperoxia. Understanding of the underlying mechanisms of this and other myocardial healing responses should prove to be instrumental in developing productive therapeutic approaches to mend the infarcted heart.

Keywords: Cardiac fibroblast; Myofibroblast; Reoxygenation; Healing

1. Introduction

Current treatment of myocardial infarction is directed to restore blood flow to the ischemic region by thrombolysis, coronary artery bypass surgery or percutaneous transluminal coronary angioplasty. Depending on the degree of success of the therapeutic intervention, the area at risk remains either hypoxic or is fully salvaged. When the area at risk remains hypoxic, the myocardial tissue loses its contractile function and becomes necrotic leading to the initiation of a wound-healing process [1]. This situation is experimentally modeled using the permanent ligation of coronary artery approach [2]. In contrast, when blood flow through the myocardium is re-established in time, hibernating myocardial tissue may regain its function but may also experience additional damage due to the reperfusion process itself. The typical clinical case of reperfusion injury occurs in acute myocardial infarction in which an occlusion of a major epicardial coronary artery is followed by re-establishment of the vascular canal of the artery. Reperfusion therapy is often used in an attempt to salvage the ischemic tissues. Other conditions involving reperfusion include cardiac surgery when the heart is arrested with cardioplegia to facilitate surgical intervention and is subsequently reoxygenated after removal of the aortic cross-clamp. Acute hypoxia, followed by abrupt reoxygenation using say cardiopulmonary bypass, results in an unintended injury mediated by oxygen free radicals [3].

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While reperfusion has been mostly studied in the context of "oxygen wastage" [4] and related oxidative injury [3], it is important to underscore that reperfusion is necessary for revival of physiological functioning and survival of ischemic tissues. Recent studies clearly establish that the challenged heart does have some inherent reparative responses ranging from recruitment of progenitor cells from the bone marrow to the injury site [5], mitosis of cardiomyocytes [6] to differentiating fibroblasts at the injury site to myofibroblast [7]. Although such naturally occurring myocardial reparative process seems to be relatively inefficient in preventing adverse functional outcomes, a clear understanding of the underlying principles of myocardial healing hold the key to successful therapy. Thus, substantial efforts have been directed towards the understanding of natural principles that underlie healing of the reperfused heart muscle. Such knowledge would serve as an indispensable tool to vitalize the natural healing responses towards improved functional outcomes. The objective of this article is to discuss the significance of oxygen-sensitive genes in the cardiac fibroblasts in post-reoxygenation myocardial healing.

2. Ischemia–reperfusion and oxygen tension

Under conditions of systemic normoxia, the heart cells receive a limited supply of O_2 not to exceed a maximum of 10% [8–10]. This is consistent with the recent observation that myocardial pO_2 in the working murine heart in vivo is 5% [11]. The myocardium may be exposed to hypoxia under a number of conditions such as transplantation, myocardial ischemia after occlusion of a major coronary artery, high altitude, and anemia. Conditions of ischemia in the heart, such as caused by the occlusion of a distal arterial vessel, result in a hypoxic area containing a central focus of near-zero O_2 pressure bordered by tissue with diminished but nonzero pO_2 (Fig. 1). These border zones extend for several millimeters from the hypoxic core, with the O_2 pressures progressively increasing from the focus to the normoxic region [12]. Moderate hypoxia is associated with a 30–60% decrease (~1–3% O_2) in pO_2 [13]. Because prior studies showed that O_2 consumption becomes O_2-limited only below 0.1% O_2 in isolated rat cardiac myocytes and below 0.3% in isolated rat hearts, physiological or metabolic changes observed in cells at 1–3% O_2 would be unlikely to result from limited oxygenation of mitochondrial cytochromes [14,15]. In response to mild or moderate compromise in pO_2, adaptive processes in surviving cells allow for physiological functioning of the tissue. These adjustments are evident for example in the hibernating myocardium where the organ maintains vital functions in the face of prolonged moderate hypoxia [16]. Although adjustments in metabolism and contractile function have been demonstrated to allow myocardial survival in the face of reduced O_2 supply, the cellular basis of such adaptation and the signaling pathways involved in the process remain to be defined [17].

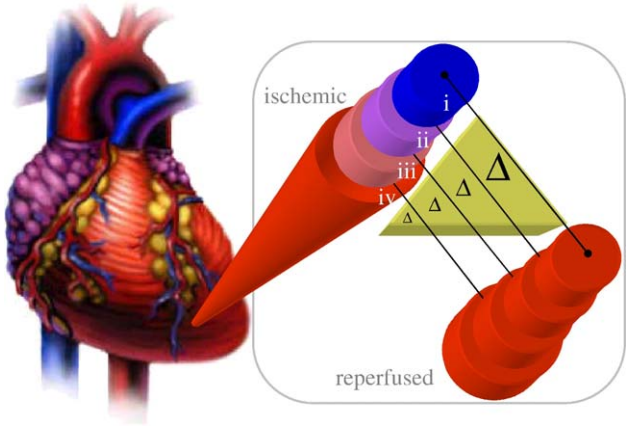

Fig. 1. Cellular responses to reoxygenation following chronic hypoxia. Chronic hypoxia results in cellular adjustments such that reoxygenation causes hyperoxic insult as evident in part in the form of oxidative damage in numerous studies. Ischemic, diagram of ischemic tissue, with focus of insult represented by the blue center (region i). Focal ischemia is known to be associated with graded oxygenation from near-zero status at focus to levels increasing with distance from focus. The concentric circles represent regions (i–iv) of the tissue with increasing graded distance from the focus of insult (blue); reperfused, diagram of the reperfused tissue with corrected pO_2 state represented by change of color from a shade of blue (hypoxic) to red. Important elements triggered by oxygen, during the course of reoxygenation-associated remodeling include: in region i cell death or fatal oxidative injury at the focal point of insult, making room for regenerating tissues; in region ii non-fatal cellular stress, triggering reparative responses; in region iii survival of phenotypically altered cells that favor remodeling (physiological or pathological/fibrogenic). Fibrosis denies room to regenerating healthy cells; and in region iv correction of pO_2 of mildly hypoxic cells localized beyond a critical distance from the focus of insult, favoring regeneration and restoration of physiological functioning of the organ. Δ, represents ΔpO_2 in response to reoxygenation. ΔpO_2: i>ii>iii>iv. While a large ΔpO_2 causes ROS mediate injury in reoxygenated region i, perceived hyperoxia supports remodeling in regions ii and iii.

3. Oxygen and oxygen sensing

Oxygen got its name from "Principe Oxygene", which means acidifying principle. "Oxy" is from Greek, and means sharp or acid; "gen" is also from Greek, and means the origin of. Taken together, oxygen means "the origin of acid". Joseph Priestly's "dephlogisticated air" [18] and Carl Scheele's [112] "fire air" were soon characterized by Antoine Lavoisier as pure respirable air [19]. Within decades of the first realization that oxygen is the element of life, Brizé-Fradin [20] noted in 1808 that "vital air" or pure oxygen would soon wear life out instead of maintaining it. That oxygen may be harmful to human health was first postulated in the late 19th century with Paul Bert's work [113] on oxygen sickness. That observation was extended through Michaeli's theoretical considerations, Gerschman's experimental verification and finally caught the interests of biomedical scientists when in 1969 McCord

and Fridovich demonstrated that a metalloenzyme produced hydrogen peroxide (H_2O_2) by combining a toxic metabolite of oxygen known as superoxide ($O_2^{\cdot-}$) with hydrogen [21,22]. Claude Bernard's claim that the "fixity of the *milieu interieur*" was essential to the life of higher organisms in the early 19th century was followed by Walter Cannon's concept of "homoeostasis" who refined and extended the concept of self-regulating mechanisms in living systems. As with numerous biological systems, the concept of homeostasis applies to the tissue biology of oxygen as well.

Cellular O_2 homeostasis is tightly maintained within a narrow range ("normoxia") due to the risk of oxidative damage from excess O_2 (hyperoxia), and of metabolic demise from insufficient O_2 (hypoxia) [23]. pO_2 ranges from 90 to below 3 Torr in mammalian organs under normoxic conditions with arterial pO_2 of about 100 Torr or ~14% O_2 [24]. Thus, "normoxia" for cells is a variable that is dependent on the specific localization of the cell in organs and functional status of the specific tissue. O_2 sensing is required to adjust to physiological or pathophysiological variations in pO_2. Whereas acute responses often entail changes in the activity of pre-existing proteins, chronic responses invariably involve O_2-sensitive changes in signal transduction and gene expression [25]. Several current articles have highlighted the key significance of understanding the fundamentals of O_2 sensing [26–36]. Current work in the field of oxygen sensing is almost exclusively focused on the study of hypoxia. Reoxygenation, on the other hand, has been mostly investigated in the context of oxidative injury and there is a clear paucity of data describing the O_2-sensitive signal transduction pathways under conditions of oxygenation that mildly or moderately exceed normoxia. During hypoxia in the heart, cells adjust their normoxic set-point such that the return to normoxic pO_2 after hypoxia is perceived as "relative hyperoxia" [25,37]. Understanding the molecular responses to such hyperoxic challenge is important because they are triggered by tissue reoxygenation in vivo. Mitochondria represent a major source of reactive oxygen species [38]. Although the majority of electrons entering the mitochondrial electron transport chain reduce molecular oxygen to water, there is evidence for "leakage" of single electrons to molecular oxygen to form $O_2^{\cdot-}$ via ubiquinone at the level of complexes I and II. This is referred to as "O_2 wastage" [4,39]. O_2 wastage is minimized by the tight coupling of the components of the electron transport system. Nevertheless, the density of mitochondria in cardiac myocytes and the high rate of oxidative phosphorylation can result in a substantial flux of $O_2^{\cdot-}$ [4]. "O_2 wastage" and oxidative injury [4] represent important aspects of ischemia–reoxygenation biology, however, it is important to underscore that reperfusion/reoxygenation is necessary for the revival of physiological functioning and long-term survival of ischemic tissues.

4. Perceived hyperoxia: p21 as a key effector

Although cells are cultured in the laboratory at an ambient O_2 concentration of 20%, which corresponds to a pO_2 of approximately 140 mm Hg at sea level, cells in the human body are exposed to much lower O_2 concentrations ranging from ~14% (100 mm Hg) in the pulmonary alveoli to 5% (35 mm Hg) in the heart. Culturing cells at room air is generally considered to be a "normoxic" condition. Studies related to cellular effects of hyperoxia have focused on concentrations of O_2 much higher than 20% [40]. A quarter of a century ago it was published in *Nature* that human diploid fibroblasts grown at 10% O_2 have a longer life than cells grown at the routine 20.6% O_2 [41]. Consistently, development of pre-implantation embryos clearly favors 7% O_2 over 20% O_2 ambience [42]. Ambient O_2 is readily dissolved in the cell culture medium [43]. Thus, isolating a primary heart cell from a 5% O_2 environment and maintaining it at 20% O_2 room-air condition may be expected to subject the cells to "O_2 stress" that would be sensed by mechanisms responding to supraphysiological levels O_2. To address this issue, recent works in our laboratory have tested the hypothesis that that O_2, even in marginal relative excess of the pO_2 to which cells are adjusted, results in activation of specific signaling pathways that alter the phenotype and function of cells [7,11,44]. We proposed that during mild hypoxia, myocardial cells adjust their normoxia set-point downward such that reoxygenation-dependent relative elevation of pO_2 results in "perceived hyperoxia" (Fig. 1).

The first line of evidence supporting the concept of perceived hyperoxia originated from in vitro studies. Adult ventricular cardiac fibroblasts, grown under conditions of 20% or 10% O_2 since isolation from 5% O_2 in vivo, proliferated significantly slower than cells grown at near-physiological 3% O_2. Growth arrest at G2/M phase was evident. On day 6 of culture, the number of cells at 3% O_2 was double compared to cardiac fibroblasts count at 20% O_2. The O_2-sensitive growth inhibition was reversible ruling out senescence as the primary route of growth arrest. Previous studies with non-cardiac fibroblasts suggest that the differentiation of fibroblasts is subject to reversal [45,46]. Cardiac fibroblasts isolated from 5% O_2 in vivo and grown at a four-fold O_2-rich ambience underwent a clear change of phenotype indicative of differentiation. Such changes were clearly minimized in cells grown under 5% O_2 culture conditions. Markers of O_2-induced differentiation of cardiac fibroblasts to myofibroblasts included substantial increase in cell size, appearance of stress fibers and parallel reorganization of smooth muscle actin with the stress fibers. Furthermore, exposure of cardiac fibroblasts to supraphysiological concentration of O_2 enhanced vimentin and smooth muscle actin expression as well as increased contractility of cells in a collagen matrix. These observations confirmed that exposure of primary adult cardiac fibroblasts to elevated O_2 triggers differentiation of the cells

to myofibroblasts. Here, "elevated O_2" refers to an ambience containing higher concentration of O_2 than the levels of O_2 to which the cells are adjusted in vivo (Fig. 2).

TGFβ1 is a known inducer of cardiac fibroblast differentiation [47,48]. The morphological/cytoskeletal characteristics of cardiac fibroblasts observed in response to elevated O_2 matched those of cardiac fibroblast cultured at 3% O_2 but treated with TGFβ1. Strikingly, inhibition of p38MAPK significantly released both TGFβ1-induced as well as elevated O_2-induced growth inhibition. These observations suggested a parallel between TGFβ1- and perceived hyperoxia-induced changes in cellular responses. Application of the high-density DNA microarray approach coupled with bioinformatics tools identified O_2-sensitive genes in cardiac fibroblasts and categorized them into functional groups [7]. Results from unbiased screening for O_2-sensitive genes confirmed the p21–p53 axis as being a key target of cardiac fibroblast exposure to elevated O_2. This finding is consistent with the current notion that many of the signaling pathways that control cellular decisions related to tissue remodeling are regulated by nuclear interactions of cell-cycle proteins [49]. Microscopic visualization of cardiac fibroblasts revealed that the nucleus of cells exposed to 20% O_2 clearly stained more prominently for the presence of p21 protein compared to fibroblasts at 3% O_2. Exposure of cardiac fibroblasts to 20% O_2 resulted in a significant increase in p21 promoter-driven luciferase reporter activity [11]. To test the significance of elevated O_2-induced p21 expression on growth arrest observed under conditions of room air, experiments were conducted using cardiac fibroblasts isolated from the heart of p21 knock-out mice. Strikingly, p21 deficient cells completely escaped from elevated O_2-induced growth arrest. Thus, p21 has been identified as a key effector of perceived hyperoxia [11].

Quantitative analysis of gene expression revealed that in addition to p21, exposure of cardiac fibroblasts to elevated O_2 resulted in the marked induction of cyclin D1, D2, G1 and Fra-2. Elevated expressions of these candidates are not only associated with growth inhibition but also with differentiation [50–57]. The D-type cyclins consist of cyclins D1, D2 and D3. Cyclin D1 synthesis is induced by p21 [58]. Cyclin D2 expression is known to be induced in multiple states of growth arrest [51]. Cyclin G1 is involved in G_2/M arrest [52]. This is consistent with the observation that cardiac fibroblasts exposed to elevated O_2 contain higher levels of cyclin G1 mRNA and are in G_2/M arrest. Fos-related antigen 2 (Fra-2) is a member of the Fos family of immediate-early genes, most of which are rapidly induced by second messengers. All members of this family act by binding to AP-1 sites as heterodimeric complexes with other proteins. However, each appears to have a distinct role. Although the role and biology of Fra-2 are less understood than those of its relatives c-Fos, Fra-1, and FosB, it is evident that elevated Fra-2 is associated with cellular differentiation [53,55,57].

Because cellular phenotype induced by perceived hyperoxia compared well with TGFβ1-induced morphological changes, the hypothesis that TGFβ1 is involved in conferring O_2-sensitive phenotype to cardiac fibroblasts was proposed. Both total and active TGFβ1 were substantially higher in cardiac fibroblasts exposed to 20% O_2. Experiments with conditioned cell culture media supported that the media of cardiac fibroblasts grown at 20% O_2 contained significantly higher amounts of active TGFβ1 compared to media from cells at 3% O_2. In support of the hypothesis that ROS can trigger the displacement of latency-associated peptide from TGFβ1, it has been observed that exposure to TGFβ1 containing conditioned

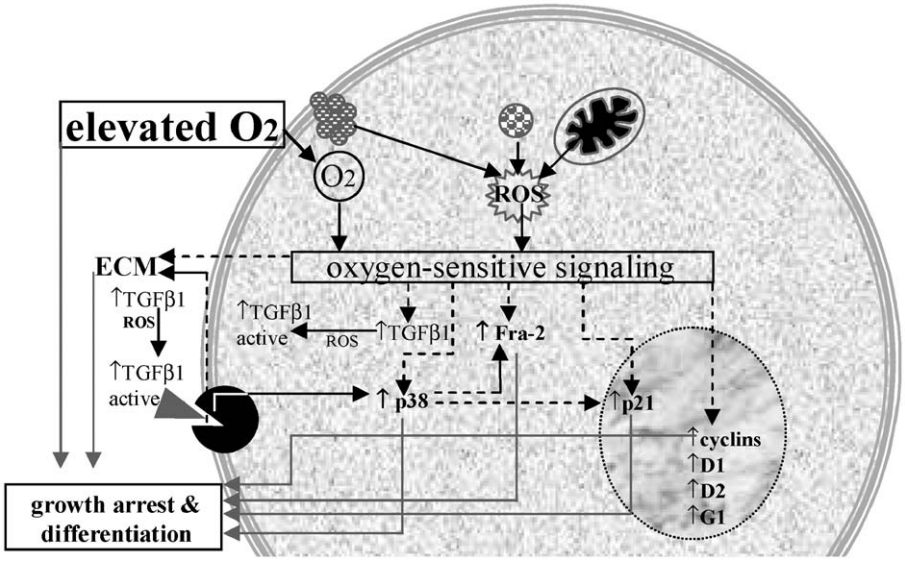

Fig. 2. "Elevated oxygen"-sensitive signaling in adult primary cardiac fibroblasts. ROS generation is enhanced followed by growth-inhibition and differentiation. TGFβ1 and downstream p38MAPK is activated. Cell cycle checkpoints known to be associated with differentiation e.g. p21, cyclins and Fra-2 are up-regulated. Broken lines represent effects where intermediary steps are expected. Grey solid lines represent association with differentiation phenotype.

culture media to oxidant challenge (UVC) significantly increased the levels of active TGFβ1. It is plausible that ROS generated by cells at elevated O_2 activate TGFβ1 [11]. p38MAPK represents a major down-stream mediator of TGFβ signaling [59–68]. Using in-gel kinase assay, it has been observed that the activation of p38MAPK in isolated primary cardiac fibroblasts is sensitive to ambient O_2. Furthermore, the growth inhibition caused by exposure to elevated O_2 can be abrogated in the presence of a p38MAPK inhibitor. These observations lead to the hypothesis that exposure of cardiac fibroblasts to perceived hyperoxia induces the activation of p38MAPK which in turn plays a significant role in executing O_2-induced growth inhibition via inducible p21 expression (Fig. 2).

5. Cardiac fibroblasts: key players in myocardial repair

In excess of 90% of the myocardium's interstitial cells are fibroblasts [69], which actively cross-talk with myocytes [70] to determine the quantity and quality of extracellular matrix [69,71,72]. Compared to myocytes, cardiac fibroblasts are relatively more resistant to oxidant insult [73]. Under certain pathological conditions such as aortic regurgitation, cardiac fibroblasts produce abnormal proportions of non-collagen extracellular matrix, specifically fibronectin, with relatively little change in collagen synthesis [74]. A characteristic feature of cardiac fibroblasts is their ability to differentiate forming myofibroblasts. Specific factors that facilitate this differentiation process have been identified [75]. Under inducible conditions, cardiac fibroblasts acquire contractile properties by irreversible acquisition of contractile proteins such as smooth muscle α-actin. The expression of smooth muscle α-actin is regulated by TGFβ1 [76]. Perceived hyperoxia triggers the differentiation of cardiac fibroblasts to myofibroblasts by oxygen-dependent mechanisms in which TGFβ1 plays a central role [11]. Reactive oxygen species modulate extracellular matrix remodeling by mediating cardiac fibroblast function and also by stimulating collagen turnover via activation of matrix metalloproteinases, enzymes critical for extracellular matrix remodeling [77]. Reactive oxygen species also stimulate the release and activation of cytokines such as TGFβ1 [11]. Recently, it has been demonstrated that the NADPH oxidase Nox 4 mediates TGFβ1-induced conversion of fibroblasts to myofibroblasts by regulating Smad 2/3 activation [78]. Antioxidants inhibit the differentiation of cardiac fibroblasts to myofibroblasts further supporting the significance of reactive oxygen species as inducers of cardiac fibroblast differentiation to myofibroblasts [79].

Cardiac fibroblasts, transfected to express the voltage-sensitive potassium channel Kv1.3, electrically couple with cardiac myocytes to contribute to the tissue's electrophysiological properties responsible for maintaining cardiac rhythm [80]. The CMG cardiomyogenic cell line, which serves as precursor of a mixture of fibroblasts and spontaneously beating cardiomyocyte-like cells, generates myocytes with sustained functionality for transplantation purposes [81]. In addition to the well-recognized co-operation with myocytes, cardiac fibroblasts are known to interact with endothelial cells to support angiogenesis in the heart [82]. Fibroblasts actively regulate interstitial fluid pressure in loose connective tissue by maintaining tension on the collagen network [83,84]. Taken together, cardiac fibroblasts represent an integral component of the key mechanisms that are required to maintain normal cardiac functioning, defend the heart against insults and orchestrate remodeling of injured tissue [85]. Cardiac fibroblasts are now viewed as a therapeutic target in heart disease [86].

It has been classically acknowledged that the mammalian heart has a very limited regenerative capacity and, hence, heals by scar formation [87], a process directly regulated by fibroblasts and their derivative matrix products. Culture of isolated myocardial cells, both myocytes and interstitial cells, have proven to be very useful over the last decade for studying molecular and cellular cardiac physiology and pathophysiology [71,88–100]. Cardiac fibroblasts are mainly responsible for the synthesis of major extracellular matrix (ECM) in the myocardium including fibrillar collagen types I and III and fibronectin. The cardiac ECM forms a stress-tolerant network that facilitates the distribution of forces generated in the heart and provides for proper alignment of cardiac myocytes. Effective reorganization of cells to regenerate an injured tissue requires the efficient laying out of a proper extracellular matrix bed. [101]. During the last two decades, the pursuit for unraveling the science of myocardial healing has developed asymmetrically with a vast majority of the studies focusing on cardiomyocytes with little or no recognition of the significance of the populous fibroblasts [102]. Addressing this oversight may hold the key to more conclusive clinical outcomes than generated from current efforts [103,104]. Indeed, in cellular therapeutics aiming at myocardial repair the delivery of mesenchymal stem cells, progenitors of all connective tissue cells including cardiac fibroblasts, to the injury site in the heart has generated favorable outcomes [105]. Human mesenchymal stem cells can be differentiated in vitro into a mixture of fibroblast and cardiomyocyte-like cells which are potentially valuable for repairing the injured myocardium [106].

One of the key determinants of the response of the cardiac fibroblast in the clinical context of myocardial damage is its transformation from a quiescent cell primarily responsible for extracellular matrix homeostasis, to an activated or differentiated cell which plays a central role in wound healing [102]. Stimulation of fibroblast proliferation may contribute to fibrosis of the heart [107]. However, cell-cycle arrest followed by differentiation to myofibroblast may support wound contraction. The first evidence for perceived hyperoxia in vivo came from a survival surgery model involving ischemia–reoxygenation of the rat heart

[7]. Examination of the post-reoxygenation tissue revealed induction of p21 accompanied by differentiation of cardiac fibroblasts to myofibroblasts in the peri-infarct region (regions ii and iii, Fig. 1). Reoxygenation represents only one of numerous factors that are associated with reperfusion. Direct evidence supporting that inducible p21 expression in the heart in vivo is indeed sensitive to $+\Delta pO_2$ came from studies demonstrating that the gene can be induced in the heart in vivo simply by transiently exposing mice to an oxygen-rich ambience [7]. p21 supports remodeling of tissues injured by oxygenation [108]. Other responses to perceived hyperoxia include TGFβ activation and differentiation of fibroblasts to myofibroblasts [11]. Both of these processes have been identified in the myocardial tissue recovering from reoxygenation injury [109,110]. Current studies identify TGFβ as an important mediator of post-reperfusion healing [111], consistent with the proposed role of perceived hyperoxia in facilitating tissue remodeling. The contribution of cardiac fibrosis as an independent risk factor in the outcome of heart failure has been evaluated [86]. Candidate drug therapies that derive benefit from actions on cardiac fibroblasts include inhibitors of angiotensin–aldosterone systems, endothelin receptor antagonists, statins, anticytokine therapies, matrix metalloproteinase inhibitors, and novel antifibrotic/anti-inflammatory agents [86]. These findings point the way to future challenges in cardiac fibroblast biology and pharmacotherapy.

That low oxygen ambience serves as a cue to trigger angiogenesis is a well-accepted notion. Studies related to perceived hyperoxia establish that the sensing of oxygen environment is not limited to hypoxia. It demonstrates that in addition to being a trigger for injury as is widely recognized, reoxygenation insult has an in-built component of tissue remodeling in the peri-infarct region induced by perceived hyperoxia. Like other reparative responses, this mechanism is also clearly inadequate to heal the heart in the case of injuries with overt clinical manifestations. Understanding of the underlying mechanisms of this and other myocardial healing responses should, however, prove to be instrumental in developing productive therapeutic approaches.

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