ANTIOXIDANTS & REDOX SIGNALING
Volume 13, Number 12, 2010
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DOI: 10.1089/ars.2010.3464

FORUM REVIEW ARTICLE

Reactive Oxygen/Nitrogen Species and the Myocardial Cell Homeostasis: An Ambiguous Relationship

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Abstract

The totality of functional cardiomyocytes and an intact cardiac progenitor cell pool are key players in the myocardial cell homeostasis. Perturbation of either one may compromise the structural and functional integrity of the heart and lead to heart failure. Reactive oxygen/nitrogen species (ROS/RNS) are important regulators of cardiomyocyte viability; more recently, the interrelation between ROS and progenitor cell behavior and fate has moved into the spotlight. Increasing evidence suggests not only that ROS participate in the regulation of cardiac progenitor cell survival but also that they likewise affect their functional properties in terms of self-proliferation and differentiation. The apparent dichotomy of ROS/RNS effects with their adaptive and regulatory character on the one hand and their maladaptive and damaging features on the other pose a great challenge in view of the therapeutic exploitation of their role in the regulation of the myocardial cell homeostasis. In this article, mechanisms and potential significance of ROS/RNS action in the regulation of the myocardial cell homeostasis, in particular with respect to the preservation of viable cardiomyocytes and the maintenance of a functional cardiac progenitor cell pool, will be discussed. *Antioxid. Redox Signal*. 13, 1899–1910.

Introduction

Heart failure is a major complication of virtually all types of cardiac diseases and a leading cause of death and hospitalization (29). Pathophysiologically, heart failure is the result of direct cardiac injury and the continuous structural and functional reorganization of the myocardium in response to it. This myocardial remodeling involves profound alterations of cardiomyocyte viability, morphology, and function (27, 61, 62, 104) as well as disturbances of the content and the organization of the myocardial matrix (101, 111). Together, these changes lead to a perturbation of the myocardial cell homeostasis in terms of a loss of functional cardiomyocytes (Fig. 1). Up until recently, the heart has been thought of as a terminally differentiated organ, therefore incapable to compensate for such a loss of functional cardiac cells. However, the recent discovery of multipotent cardiac progenitor cells with the capacity to differentiate into all cardiac cell lineages, including functional cardiomyocytes (Fig. 2), has led to a shift in paradigm (7). It is broadly accepted now that resident cardiac progenitor cells represent a source of newly formed cardiomyocytes, hence maintaining the myocardial cell homeostasis over a lifespan (7) (Fig. 1). Functional impairment of these cardiac progenitor cells has detrimental effects on the structural and functional integrity of the heart and may lead to heart failure. Similarly, if the loss of functional cardiomyocytes outbalances the intrinsic regenerative capacities of the heart, heart failure may develop despite of an intact cardiac progenitor cell pool.

Reactive oxygen species (ROS) and nitrogen species (RNS) are key mediators of myocardial remodeling (96) and therefore contribute either directly or indirectly to the regulation of the myocardial cell homeostasis. Whereas mostly considered damaging in the past, there is now an increasing body of evidence in support of a role for ROS/RNS in cardioprotection and repair. These recent developments changed the view of such species as purely detrimental contributors to myocardial remodeling and highlighted the need for a more comprehensive understanding of the roles of cardiac ROS/RNS, their sources, and their potential targets. In this review, we discuss the potential significance of ROS/RNS in the regulation of the myocardial cell homeostasis, in particular with respect to the preservation of viable cardiomyocytes and the maintenance of a functional cardiac progenitor cell pool.

The Cellular Redox Equilibrium

ROS are highly reactive oxygen moieties that arise from incomplete reduction of molecular oxygen (O_2) either by leak of electrons from the mitochondrial respiratory chain or through action of intracellular oxidases, including NADPH oxidase (NOX) and xanthine oxidase. A number of

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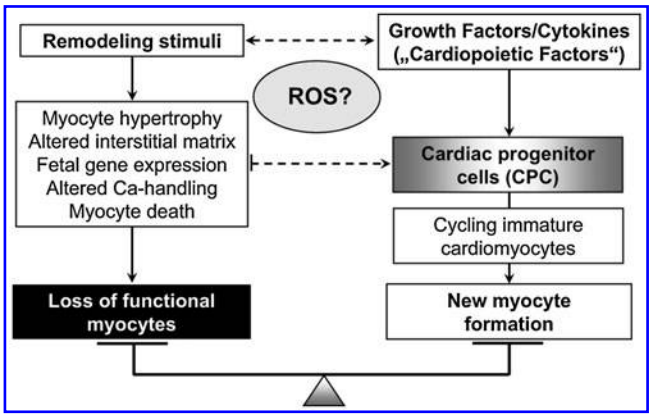

FIG. 1. Cardiac cell homeostasis. The myocardial cell homeostasis is determined by the totality of functional cardiomyocytes on the one hand and the formation of new myocytes on the other (for details see text). There may exist an interrelation between myocardial remodeling and cardiac progenitor cells in terms that remodeling stimulates cardiac progenitor cell differentiation, whereas cardiac progenitor cells may affect the myocardial remodeling process, namely, cardiomyocyte survival, in a paracrine way.

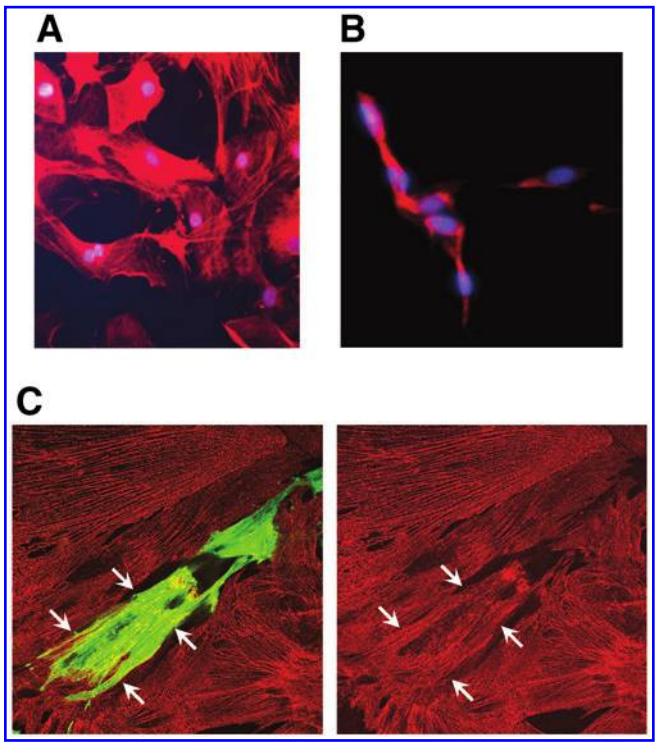

FIG. 2. Differentiation of cardiac progenitor cells. Cardiac progenitor cells from mouse hearts differentiated into **(A)** smooth muscle cells (red staining: tropomyosin) and **(B)** endothelial cells (red staining: van willebrand factor). **(C)** Cardiac progenitor cell (arrows) from a green fluorescent protein-expressing mouse heart (green: green fluorescent protein, *left panel*), which differentiated into a cardiomyocyte that is structurally indistinguishable from the surrounding neonatal rat ventricular myocytes (*right panel*; red staining: α-actinin). (Pfister O and Liao R, unpublished data).

antioxidants are in charge to remove excessive ROS and to maintain a physiological redox balance (37) (Fig. 3). Various remodeling stimuli (*e.g.*, neurohormones, growth factors, and cytokines) that are released in response to cardiac injury enhance ROS production by activating ROS-generating enzymes and/or decrease the antioxidant defense capacities, which results in a net increase of ROS (oxidative stress) (16, 17, 42). Excessive ROS may directly induce cellular injury *via* oxidation of DNA, lipids, and proteins associated with cell death, disease, and premature aging. ROS also participate in cell signaling through activation of redox-sensitive signaling cascades. Thereby, they initiate both protective (adaptive) and damaging (maladaptive) cellular events. According to the redox-homeostasis model depicted in Figure 3, the following three strategies to modify oxidative stress-associated processes can be delineated: (i) scavenging or neutralization of ROS by enhancing antioxidant capacities, (ii) inhibition of sources of ROS, and (iii) protection of potential targets from oxidation.

A Delicate Balance Between Oxidative and Reductive Stress

Markers of oxidative stress are elevated systemically and locally in the myocardium in animal models and humans with heart failure (18, 47, 51, 53, 63, 93). This notion has led to the hypothesis that ROS play an active role as mediators of myocardial remodeling. An array of studies showing that antioxidant treatment or inhibition of ROS-generating oxidases improved remodeling in cardiomyocytes *in vitro* (76, 112) and in rodent hearts *in vivo* supported this hypothesis. In mice, for instance, dimethylthiourea mitigated oxidative stress and prevented postmyocardial infarction remodeling and heart failure (49), and the antioxidant N-2-mercaptopropionyl glycine diminished hypertrophic remodeling due to pressure overload in animals subjected to transverse aortic

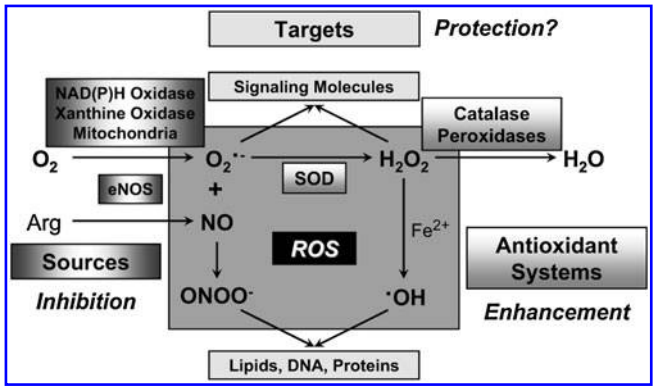

FIG. 3. Cellular redox balance. Reactive oxygen and nitrogen species are produced by a variety of sources (NADPH oxidase, xanthine oxidase, mitochondria, and nitric oxide synthase) and degraded through action of a number of antioxidant systems (superoxide dismutases, catalase, and peroxidases). Perturbation of the cellular redox balance leads to impaired cell signaling or direct cellular injury going along with cell death, disease, and premature aging. Therapeutic strategies to counterbalance oxidative stress may include enhancement of antioxidant capacities, inhibition of sources of reactive oxygen species (ROS), or protection of ROS targets.

constriction (15). Similarly, inhibition of the xanthine oxidase using oxypurinol or allopurinol improved remodeling after myocardial infarction in mice (19) and in the spontaneously hypertensive heart failure rat model of dilated cardiomyopathy (72). Encouraged by the beneficial effects of such treatments in animal models of heart failure, a multitude of human studies were designed to translate these findings into a clinical setting of patients suffering from—or at risk for—cardiovascular disease. Disappointingly, however, the vast majority of these studies yielded negative results. Namely, the antioxidant vitamin α-tocopherol and the provitamin β-carotene failed to reduce cardiovascular morbidity and mortality in large clinical trials, whereby some studies even reported an increased incidence of cardiovascular death and/or heart failure (40, 60, 87, 115). Similarly, the recently published Oxypurinol Therapy for Congestive Heart Failure trial (39), which investigated the effect of the xanthine oxidase inhibitor oxypurinol in patients with symptomatic heart failure, turned out negative in the all-over, unselected patient population. There was even an adverse trend regarding cardiovascular death and heart failure hospitalization in the active treatment group (13), although a *post hoc* analysis suggested a benefit for the subpopulation of patients with elevated serum uric acid levels. Besides patient selection, other factors such as timing, inappropriate end-points, antioxidant effects of adjuvant therapies, and inappropriate antioxidants may explain why such treatment strategies were ineffective in these trials. However, there are alternative explanations for the lack of beneficial effects of antioxidants. First, under certain circumstances, ROS are protective rather than deleterious. Second, reductive stress too can inflict damage to the heart: Rajasekaran *et al.* (86) recently described oxido-reductive stress in mice with a cardiac-specific expression of a mutant of the human small heat shock protein α-B-crystallin (hR120GCryAB), which exhibit a distinct form of cardiomyopathy associated with protein aggregation. The reductive stress in these mice was due to the enhanced expression and activity of intrinsic antioxidants, leading to an increase in reduced glutathione. Interestingly, in an earlier study, Maloyan *et al.* demonstrated enhanced apoptosis in the hearts of hR120GCryAB mice (64). Taken together, these observations suggest that not only oxidative, but similarly reductive stress can disturb the myocardial cell homeostasis causing cardiomyopathy and heart failure. Figure 4 shows a proposed model for the redox continuum and its potential (patho-)physiological implications in the cardiomyocyte. According to this model and similar to the regulation of the intracellular acid–base equilibrium with its well-defined, physiological pH value, an optimal redox state of the cardiomyocytes may be required for the heart to maintain its structural and functional integrity.

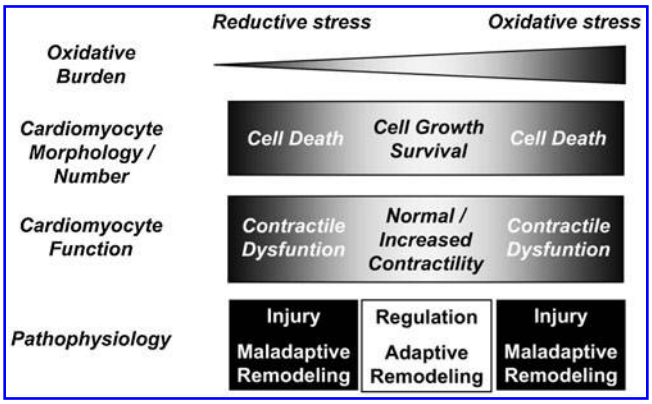

FIG. 4. The redox continuum and its (patho-)physiological implications. A change in the oxidative burden can affect the cardiomyocyte phenotype in terms of viability, morphology, and function. An increase in oxidative load (oxidative stress) shifts the cell from a regulatory program (cell growth and survival, maintained or enhanced contractile function) toward injury (cell death, contractile dysfunction). Similar changes have recently been described for an unphysiological decrease in oxidative load (reductive stress).

ROS as Regulators of Cardiomyocyte Death and Survival

ROS-associated proapoptotic effects

The role of ROS in cell damage and apoptotic cell death is well documented. Besides their nonselective effects associated with the oxidation of structural and functional molecules, including lipids, proteins, and nucleic acids, that lead to cell damage and death, ROS can selectively activate intracellular signaling cascades that are linked to programmed cell death. This ROS-mediated proapoptotic signaling has been extensively reviewed by others (23, 68). In brief, such signaling includes the ROS-dependent activation of ASK-1, p38, and c-Jun-N-terminal kinase (43, 90), which play an important role in stress-induced apoptosis in the heart (114) and are responsible for the regulation of downstream targets such as the Bcl-2 family proteins, p53 and caspases [see review (68)]. Besides this activation of proapoptotic signaling, ROS can directly trigger the mitochondrial death pathway in a process referred to as "ROS-induced ROS release," which describes the ROS-induced oxidative burst that originates from the mitochondrial electron transport chain and is associated with the dissipation of the mitochondrial membrane potential, an event generally believed to be a point-of-no-return in cell death [see review (35)].

ROS-associated prosurvival signaling

In addition to and generally in parallel to the beforementioned proapoptotic signaling, ROS mediate the activation of the mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) and the phosphoinositide 3-kinase (PI3K)/Akt pathways. Both of these pathways have been implicated in cell growth and survival in various cell types, including cardiomyocytes. They exhibit a variety of individual and common activators such as cytokines and growth factors, including agonists of the Gq-protein-coupled receptors.

Activation of the MEK/ERK pathway is initiated by the receptor-mediated activation of the small G-protein Ras and translocation of Raf-1 to the plasma membrane. This Ras-Raf activation is followed by the downstream phosphorylation and activation of MEK1/2 and ERK1/2. ERK1/2, in turn, activate an array of transcription factors that are in control of the expression of genes involved in the regulation of cell growth, proliferation, survival, and differentiation (Fig. 5). Similar to other mitogen-activated protein kinase pathways, the MEK/ERK pathway can be activated by ROS of either exogenous (*e.g.*, applied hydrogen peroxide) (55) or endogenous

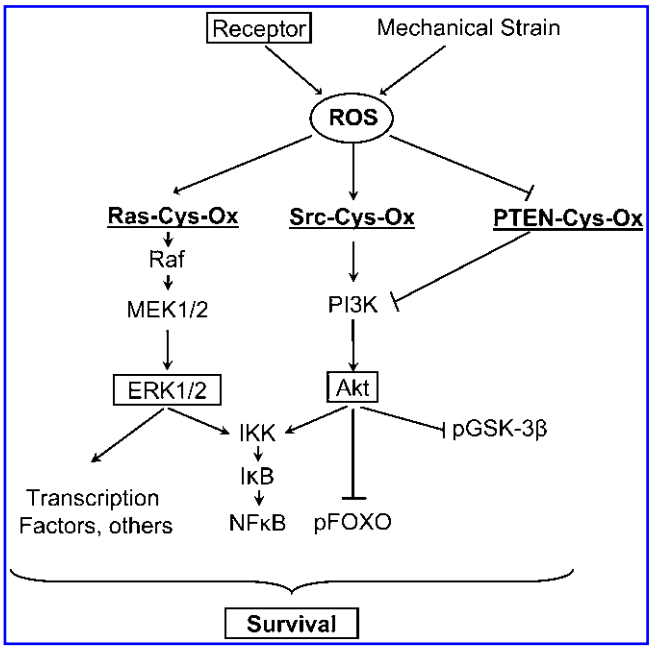

FIG. 5. ROS signaling in cardiomyocyte survival. Pathways involved in the regulation of cardiomyocyte survival may include the mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) and the phosphoinositide 3-kinase (PI3K)/Akt pathway. Both of these pathways can be activated by ROS through post-translational oxidative thiol modification of cysteine residues on upstream regulators, including Ras, Src, and phosphatase and tensin homolog (PTEN). Downstream effectors include glycogen synthase kinase (GSK)-3 as well as a variety of transcription factors. Arrows indicate activation, inverted T-bars indicate inhibition.

origin (*e.g.,* produced in response to receptor stimulation) (107), with Ras acting as a direct ROS target. Ras exhibits four cysteine residues, which can be oxidatively modified and oxidative thiol modification of Cys118 has been implicated in Ras activation (57). In fact, oxidative modification of Ras thiols has recently been confirmed in the ROS-dependent prohypertrophic signal transduction in cardiomyocytes in response to Gq-protein-coupled receptor stimulation (54) and mechanical strain (83). The MEK/ERK pathway is also an important mediator of cell survival as previously described in other cell types (36) as well as more recently in cardiomyocytes (10, 55). Bueno *et al.* demonstrated activation of ERK1/2 in cardiac-specific MEK1 transgenic mice and in adenoviral-mediated MEK1-overexpressing neonatal rat ventricular myocytes, which was associated with partial protection against apoptosis. Further, MEK1 transgenic mice developed compensated hypertrophy without progression to ventricular dilation and failure (10). Kwon *et al.* confirmed these findings in adult rat ventricular myocytes and linked the activation of ERK1/2 to oxidative stress. They showed that the ROS-dependent activation of ERK1/2 in response to exogenous hydrogen peroxide in cardiomyocytes is antiapoptotic, as hydrogen peroxide-associated cardiomyocyte apoptosis was markedly enhanced in the presence of the MEK1/2 inhibitor U0126 (55). Taken together, these observations provide solid support for a role of ROS-mediated ERK1/2 activation in cardiomyocyte survival.

The probably more classical mediator of cell survival is the PI3K/Akt pathway (12). In cardiomyocytes, Akt is activated in response to stimulation of a variety of receptors that are associated with cardioprotection, including Gq-protein-coupled receptors (75), insulin- (4) and insulin-like growth factor 1-linked tyrosine kinase receptors (50, 59), the erbB receptor system (25), and gp130-associated receptors (78). Activation of PI3K/Akt inhibits apoptosis in cardiomyocytes exposed to hypoxia *in vitro* (24, 66) and in rodent hearts after ischemia-reperfusion *in vivo* (24, 67). Similar to the MEK/ERK pathway, Akt can be activated by ROS, and ROS-mediated Akt activation has been implicated in cell survival (55, 110). Wang *et al.* found that Akt was activated in response to exogenous hydrogen peroxide and that this activation mitigated oxidative stress-associated apoptosis in various nonmyocyte cell lines (110). Similar findings were obtained by Kwon *et al.* in cultured rat cardiomyocytes (55). Whereas ROS-dependent activation of the MEK/ERK pathway occurs through direct interaction of ROS with Ras as described above, the mechanisms of ROS activation of the PI3K/Akt pathway are less clear. Recently, oxidative inhibition of the phosphatase and tensin homolog PTEN, a phosphatase known to interfere with PI3K signaling, has been implicated in ROS-dependent Akt-activation (58). Additional mechanisms may include the oxidative modification of other upstream regulators of Akt, such as Src (20, 28) or other protein tyrosine kinases. Endothelial nitric oxide synthase (eNOS), NFκB, FOXO3a, and glycogen synthase kinase-3β have all been identified as downstream targets of Akt [see review (9)] and their Akt-dependent regulation represents an inherent feature of the progrowth and prosurvival properties of Akt. Namely, FOXO3a (98) and glycogen synthase kinase-3β (45, 70), which are both inhibited in response to Akt activation, have recently been characterized as downstream effectors of Akt in cardiomyocytes (Fig. 5).

Amount and species as determinants of ROS effects

The significance of oxidative events depends on an array of circumstances, including the nature and intensity of the stimulus, the amount and species of the involved ROS, the basal redox state of the cell or tissue, the duration of ROS exposure, the specific source of ROS, the localization (extracellular *vs.* intracellular) and the intracellular compartmentalization of ROS, the timing and the dynamics of ROS release, as well as the available antioxidant defenses. It is generally appreciated that low concentrations of ROS are associated with rather adaptive and/or protective processes, including cell growth and survival, whereas high concentrations lead to cell damage and death. In a simplified *in vitro* cardiomyocyte model of exogenously applied ROS, for instance, hydrogen peroxide induced differential, concentration-dependent activation of specific kinase signaling pathways resulting in hypertrophy in response to low ($<50\,\mu M$) and apoptosis to high concentrations ($\geq 100\,\mu M$) (55). Besides the quantity of ROS, the quality of oxidative stress may likewise play a role, as different species may exert distinct effects. In a recently completed study in humans, we found that standard heart failure treatment targeting the renin-angiotensin system regulated copper-zinc superoxide dismutase (Cu/Zn-SOD) in the cardiac circulation and that this occurred in the absence of a change in net oxidative stress in our setting (53). Cu/Zn-SOD

dismutates superoxide into hydrogen peroxide. This may leave net oxidative stress unaffected, but the change in the oxidative stress substrate may, nevertheless, be important. Superoxide readily reacts with NO, which leads to the formation of the highly damaging radical peroxynitrite while decreasing the bioavailability of NO, a molecule that exerts beneficial effects in the cardiovascular system (5). When mimicking the phenotype observed in the coronary sinus blood of our heart failure patients *in vitro*, we found that cardiomyocytes with adenoviral-mediated overexpression of Cu/Zn-SOD exhibited higher levels of phosphorylated Akt under basal conditions, and a more pronounced increase in response to stimulation as compared to control-infected cells (Fig. 6) (Häuselmann *et al.*, unpublished observation). These observations suggest that hydrogen peroxide rather than superoxide has the potential to activate the PI3K/Akt pathway in cardiomyocytes, which is consistent with what has been described in other cell types [see review (34)].

NO and RNS

NO is formed by the conversion of arginine to citrulline through action of the NOS family. At least three isoforms of NOS exist: neuronal (nNOS or NOS1), inducible (iNOS or NOS2), and eNOS (eNOS or NOS3). All three isoforms are expressed in cardiomyocytes, whereby expression of iNOS is induced by various cytokines (74). Binding of NO to the heme part of guanylyl cyclase leads to the formation of cyclic guanosine monophosphate, which acts as second messenger of NO-mediated signaling. In addition, NO induces posttranslational modifications of protein tyrosine (tyrosine nitration) and cysteine residues (cysteine nitrosylation), thereby altering target protein function. The abundance of nitrotyrosinylated protein is increased under pathological conditions in the heart (51, 88) and NO-dependent posttranslational modifications of proteins involved in calcium handling can affect cardiomyocyte calcium homeostasis and contractile performance (38, 48, 103, 113). At high concentrations, NO reacts with superoxide to form peroxynitrite ($ONOO^-$), a damaging moiety responsible for lipid peroxidation and irreversible protein modifications that are usually associated with cell damage.

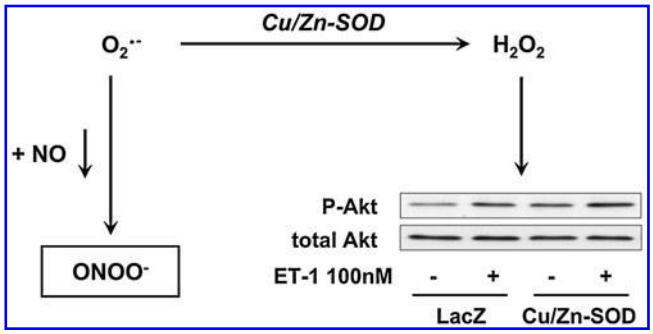

FIG. 6. The oxidative stress substrate. The substrate of oxidative stress may matter, as distinct ROS may exert distinct effects. Superoxide readily reacts with nitric oxide—thereby reducing its bioavailability—to form peroxynitrite. In contrast, cardiomyocytes overexpressing copper-zinc superoxide dismutase (Cu/Zn-SOD) exhibit more pronounced phosphorylation of Akt under basal conditions and after stimulation as compared to control-infected cells (Häuselmann *et al.*, unpublished observation). Taken together, a shift from superoxide to hydrogen peroxide could be associated with a survival benefit. ET-1, endothelin 1.

Roles of NO in cardiomyocyte death/survival

The effects of RNS depend on a variety of factors, including source and amount as well as localization and timing of their production. Similar to ROS, high amounts of NO, as they can be produced by iNOS, mostly exert proapoptotic effects. Accordingly, a series of *in vitro* studies in cultured cardiomyocytes has implicated iNOS-derived NO in cytokine-induced cardiomyocyte apoptosis. Arstall *et al.* showed that interleukin-1-β and interferon gamma induced iNOS expression, nitrite production, and apoptosis in neonatal rat cardiomyocytes, whereby the latter could be inhibited by the iNOS inhibitor 2-amino-5,6 dihydro-6-methyl-4H-1,3-thiazine (2). Increased expression of iNOS and enhanced NO production associated with cardiomyocyte apoptosis in response to cytokine exposure were also shown by Ing *et al.* and Song *et al.* in neonatal (44, 100) and Pinsky *et al.* in adult cardiac myocytes (84). In contrast to ROS, however, only little is known about NO-dependent downstream signaling, although enhanced expression of p53 and shifts in the Bax/Bcl-2 ratio have been implicated in the proapoptotic effects of iNOS-derived NO.

Consistent with these *in vitro* findings, *in vivo* lack of iNOS improved contractile performance and survival early (30 days) (21) and decreased the amount of apoptotic cardiomyocytes late (4 months) after myocardial infarction in mice (94), hence supporting a role for iNOS in adverse postmyocardial infarction remodeling. More recently, iNOS-derived NO has also been implicated in maladaptive hypertrophic remodeling as cardiomyocyte apoptosis was decreased and survival improved in iNOS-knock out as compared to wild-type mice in a model of calcineurin-induced cardiac hypertrophy (99). In contrast, mostly anti-apoptotic effects have been described in connection with NO derived from eNOS. In particular, lack of eNOS increased apoptosis in neonatal hearts (22) and worsened post-myocardial infarction remodeling in terms of left-ventricular dimensions and contractile function in mice (97).

NO may play an even more complex role as mediator of agonist-associated antiapoptotic effects. Das *et al.* recently demonstrated that the antiapoptotic effect of the phosphodiesterase-5 inhibitor sildenafil was diminished in eNOS-knock out, but—intriguingly—absent in iNOS-knock out cardiomyocytes, hence suggesting a protective role not only of eNOS- but also of iNOS-derived NO in cardiomyocyte survival in an agonist-associated setting (14).

Roles of ROS/RNS in Cardiomyocyte Growth and Contractile Function

We focused on the roles of ROS/RNS in the maintenance of the myocardial cell homeostasis in terms of preservation of cardiomyocyte survival, whereby special attention was paid to the kinase-driven pathways. Other mechanisms not discussed in this article include the regulation of redox-sensitive transcription factors and/or other regulatory proteins. Further, the functional integrity of viable cardiomyocytes is no less important. There is a solid body of evidence in support of an important regulatory role of ROS/RNS in excitation–contraction coupling, mostly *via* oxidative and nitrosative modifications of various calcium channels, including L-type

calcium channels (11, 103), ryanodine receptor (6, 105), sarcoendoplasmic reticulum calcium ATP-ase (SERCA2a) (52, 56), and sodium-calcium exchanger (NCX) (30, 89), the discussion of which would be beyond the scope of this article [for recent reviews, see (31, 116)]. Finally, ROS and RNS play a pivotal role in the regulation of cardiomyocyte hypertrophy as previously reviewed in this journal (92, 102).

Cardiac Progenitor Cells

First identified in highly regenerative tissues such as the bone marrow and liver, organ-specific progenitor cells have meanwhile been found in virtually all types of organs, including such with a high level of differentiation and slow turnover as the lungs, the central nervous system, and the skeletal muscle. They contribute to the cellular tissue homeostasis and are a source of cellular regeneration following injury. Whereas the heart has long been considered a strictly postmitotic organ, incapable to replenish its cell pool, a shift in paradigm occurred when immature cycling cardiomyocytes were detected in the human heart following myocardial injury (8). More recently, cardiac progenitor cells resident in adult myocardium and capable of functional differentiation into cardiomyocytes were identified (7). These progenitor cells represent a likely source of such immature cardiomyocytes. Hence, cardiac progenitor cells are important players in the myocardial cell homeostasis, and impairment of cardiac progenitor cell survival or function may profoundly affect the structural and functional integrity of the heart.

The cardiac side population

The stemness of a cell is not linked to a single-specific marker, but rather defined by a combination of a variety of stem cell-associated cell surface markers as well as phenotypic and behavioral properties. One of these properties consists of the ability to extrude vital dyes, including Rhodamine 123 and Hoechst 33342, from the cell (32, 41, 71). This phenomenon is mediated by the ATP-binding cassette (ABC) transporters Mdr1 and Abcg2 and offers the possibility to isolate these cells according to their Hoechst content upon incubation with the DNA-binding dye Hoechst 33342. Their appearance as "Hoechst low cells" in the low-red, low-blue zone of the Hoechst fluorescence-assisted cell sorting dot plot readout, aside from the "Hoechst high" main population, rendered them the name "side population" (SP) (32). Such SP cells have first been identified and characterized in murine bone marrow cell suspensions (32) and their phenotypic analysis demonstrated great enrichment in hematopoietic stem cell surface markers including c-kit, stem cell antigen-1, and Mdr1. Importantly, the vast majority of hematopoietic stem cell activity in the bone marrow could be attributed to SP cells, as evidenced by a 1000-fold increase in *in vivo* reconstitution activity in competitive repopulation experiments. Meanwhile, SP cells have been isolated from a variety of solid organs, including the heart (41, 80) (Fig. 7, Lorenz and Häuselmann). Specification of the phenotype of these cardiac SP cells showed marked differences between cardiac and bone marrow SP cells in terms of hematopoietic surface markers with virtually no expression of CD45 and CD34 in SP cells from cardiac origin, but great similarity in expression of the stem cell-specific marker stem cell antigen-1 (80). We further assessed the *in vitro* differentiation capacities of these cells by coculturing cardiac SP cells isolated from green-fluorescent mice with rat ventricular cardiomyocytes. In these studies, solid cardiomyogenic differentiation of SP cells in structural and functional respect was achieved (80). Taken together, these observations underscore that cardiac SP cells represent a unique, heart-resident progenitor cell population.

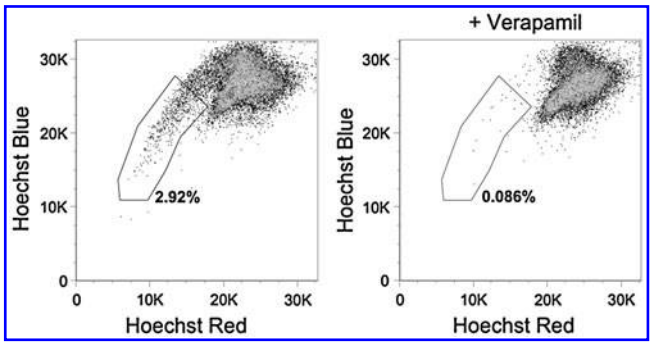

FIG. 7. Cardiac side population cells isolated from adult mouse hearts. Fluorescence-assisted cell sorting analysis of a cardiomyocyte-depleted cell suspension stained with Hoechst 33342 shows the characteristic pattern of Hoechst-extruding cells appearing in the "low-red," "low-blue" zone (gated area) aside from the main population. Verapamil, a calcium antagonist, inhibits the calcium-dependent ATP-binding cassette (ABC) transporters responsible for the Hoechst efflux, prompting the side population to disappear.

ABC Transporters as Regulators of Cardiac Progenitor Cells

ABC transporters feature an ABC region to hydrolyze ATP that supplies the energy required for exportation of cytotoxic substrate against steep concentration gradients from the intra- to the extracellular compartment. The ABC transporter proteins Abcg2 and Mdr1 are highly enriched in cardiac progenitor cells, and whereas their activity is the major determinant of the SP phenotype, they may likewise play an active role in the regulation of cardiac progenitor cell behavior and fate. We have recently shown that cardiac SP cells isolated from Abcg2-deficient mice show impaired proliferation and survival, whereas overexpression of Abcg2 improved survival and enhanced proliferation, but limited the differentiation capacities of cardiac SP cells (81). These findings suggest that Abcg2 plays a major role in the maintenance of an intact cardiac progenitor cell pool.

Role of Abcg2 in the oxidative stress response

Cardiac progenitor cells are exposed to an array of stressors, including hypoxic and oxidative stress. Analyzing the transcriptome of these cells, Martin *et al.* identified a distinct expression pattern inherent to SP cells that includes cytoprotective factors associated with the oxidative stress response (65). Using gain-of-function studies, they found that Abcg2 induced low-grade oxidative stress as reflected by a decrease of the reduced to oxidized glutathione (GSH/GSSG) ratio that was associated with an increase of the antioxidant α-glutathione reductase and upregulation of the oxidative stress program in C2C12 SP cells. Further, overexpression of Abcg2 ameliorated survival of mouse embryonic fibroblasts in response to hydrogen peroxide. These findings suggest that

Abcg2 acts as a regulator of the oxidative stress pathway and confers partial resistance against oxidative cell damage *via* a mechanism that involves ROS. With regard to cardiac SP cells and consistent with these observations, we found enhanced apoptosis in Abcg2-deficient as compared to Abcg2-competent wild-type cardiac SP cells when exposed to hydrogen peroxide (81), hence confirming that Abcg2 enhances oxidative stress tolerance and diminishes oxidative stress-associated cell damage in cardiac progenitor cells. Still, the precise mechanism whereby Abcg2-evoked ROS activate the oxidative stress program and promote cell survival remains to be elucidated. Similar to as described before for ROS-dependent prosurvival signaling in cardiomyocytes and to what is known from ROS-mediated ischemic preconditioning, this process may involve ROS-dependent activation of different signaling kinases, including the MEK/ERK and the PI3K/Akt pathway and/or the regulation of specific transcription factors (33, 79). A model for the Abcg2-mediated ROS-associated effects as reported by Martin *et al.* (65) and Pfister *et al.* (81) is given in Figure 8.

Cardiac Progenitor Cells in Myocardial Injury

Whereas the physiological cell turnover may be considered low in a highly differentiated and low-proliferative organ as the heart, the instantaneous loss of a large amount of viable, functional cells as it occurs in response to injury can impose high demands on the regenerative capacities of the organ-specific progenitor cell pool. Using a mouse model of myocardial infarction, we investigated how the heart is organized to meet such a demand and studied the potential role of bone-marrow-derived stem cells. We found that the latter contributed little to the maintenance of the cardiac cell homeostasis during normal postnatal growth and adulthood. In contrast, after myocardial infarction, the cardiac progenitor cell pool was acutely depleted, but became subsequently replenished over time in a process that encompassed both enhanced self-proliferation of resident cardiac progenitor cells and mobilization and selective homing of bone-marrow-derived stem cells (73). These observations suggest that bone-marrow-derived stem cells may play a role in the maintenance of the cardiac cell homeostasis in response to cardiac injury.

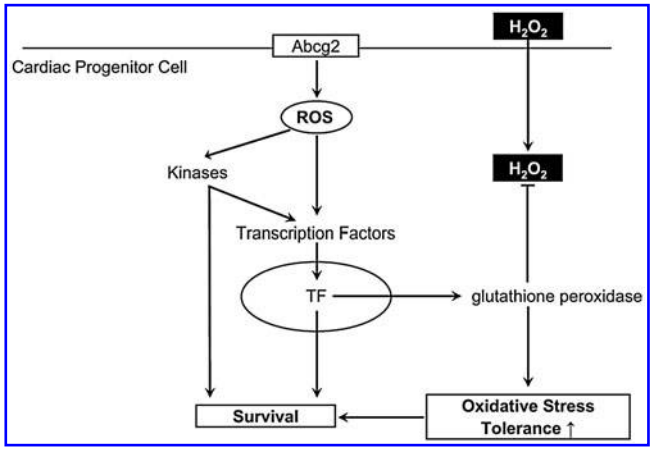

FIG. 8. Model for Abcg2-mediated ROS-associated effects. Abcg2 induces low-level oxidative stress that activates the oxidative stress program. Protective factors activated within the scope of this program may include antioxidants that contribute to an enhanced oxidative stress tolerance conferring partial protection against oxidative stress-associated cell death.

Potential Role of ROS in Progenitor Cell Survival and Function

Oxidative stress is a major feature of myocardial infarction, but knowledge on the interrelation of ROS and cardiac progenitor cells is scarce. Interestingly, recent work linking ROS with cardiac progenitor cells suggests a similar dichotomy of ROS action as it can be observed in other cell types. In an *in vitro* and *in vivo* model of diabetes, a condition associated with oxidative stress, Rota *et al.* found low levels of ROS and a low degree of DNA damage in cardiac progenitor cells undergoing DNA repair and replication, whereas a higher oxidative burden was detectable in apoptotic or necrotic cells (91). Similar findings were reported for endothelial progenitor cells, where exogenous hydrogen peroxide induced apoptosis (108) and endothelial progenitor cells deficient in the antioxidant glutathione peroxidase-1 exhibited impaired migratory capacities and proangiogenic competence that were attributable to oxidative stress (26). Similar pathways as described in cardiomyocytes, namely the PI3K/Akt pathway, mediate progenitor cell survival (108) and PI3K/Akt as well as eNOS and vascular endothelial growth factor have been identified as positive stimuli of endothelial progenitor cell mobilization (106). All of these factors are connected to ROS, as phosphorylation of Akt (see above) and eNOS may depend on ROS (3) and vascular endothelial growth factor *per se* signals through ROS (109).

How ROS affect cardiomyocyte differentiation of cardiac progenitor cells remains to be established. However, ROS have been implicated in cardiomyogenic lineage commitment and differentiation of embryonic stem cells. Sauer *et al.* (95) were the first to suggest a role for ROS in cardiac cell differentiation in embryonic stem-cell-derived embryonic bodies. In these studies, application of 10 n*M* hydrogen peroxide or the ROS generator menadione induced cardiac differentiation within the embryonic body. In contrast, high levels of hydrogen peroxide (100 μM) as used by another group of investigators impaired cardiac cell differentiation of embryonic stem cells (85). Further, forced generation of ROS *via* overexpression of a constitutively active Rac1, the regulatory small GTPase subunit of NOX, markedly inhibited cardiac cell differentiation of embryonic stem cells, whereas a constitutively active Rac1 mutant, which did not activate NOX, had no such effect. However, expression of the same constitutively active Rac1 in embryonic stem-cell-derived cardiomyoblasts, that is, at a later stage of the differentiation process, facilitated their differentiation into mature, beating cardiomyocytes. Taken together, these observations implicate that the effects of ROS depend on certain basic conditions, which at least include the ROS concentration and the developmental stage of the cell, whereby the latter may affect the basal redox state and/or the oxidative stress responsiveness.

The Progenitor Cell Redox Status

Piccoli *et al.* found that bone-marrow-derived hematopoietic progenitor cells constitutively produce low levels of

ROS (82). Although the authors of this study did not provide data on the possible function of these ROS, previous work reviewed by Noble *et al.* (77) proposed that cells that are slightly more oxidized show a greater response to inducers of differentiation or cell death, but less response to inducers of proliferation or survival, whereas slightly more reduced cells are more responsive toward inducers of cell survival and division and less toward inducers of differentiation or death. The observations by Piccoli *et al.* (82) are reminiscent of the beforementioned findings by Martin *et al.*, who showed that Abcg2-expression in C2C12 SP cells is associated with low-grade oxidative stress (65). What this slightly oxidized state of Abcg2-expressing SP cells means with regard to their self-proliferation and differentiation capacities remains to be established.

ROS as Regulators of Cardiac Progenitor Cell Senescence

Besides the regulation of cell survival, oxidative stress induces cardiac progenitor senescence, thereby affecting status and functionality of the cardiac progenitor cell pool and diminishing its regenerative capacities. As previously reviewed in this journal, mechanisms involved in ROS-dependent cardiac progenitor cell senescence include the modulation of telomerase activity and the control of telomeric length as well as the regulation of the cell cycle inhibitors p16INK4a and p53 [see review (46)].

Role of NO in the Regulation of Cardiac Progenitor Cells

NO has emerged as an important regulator of endothelial progenitor cells, where eNOS-derived NO mediates progenitor cell mobilization (1). In contrast, knowledge on the role of NO in resident cardiac progenitor cells is scarce. Recently, McMullen *et al.* provided evidence that NO acts as an inhibitory force of the functional specification of developing ventricular cells. They showed that NO prevented the development of proper calcium transients in cardiac progenitor cells of the embryonic heart (69). However, cardiac progenitor cells from embryonic hearts exhibit distinct properties, and therefore these findings cannot be extrapolated to adult cardiac progenitor cells, in which the roles of NO remain to be investigated.

Summary and Conclusion

ROS and RNS are important players in the matter of life or death of the cell, whereby their contribution can be in favor of either one. More recently, the potential significance of ROS in the regulation of cardiac progenitor cell behavior and fate has moved into the spotlight. Much more research is needed until we are able to understand the precise roles of ROS/RNS in the regulation of the myocardial cell homeostasis. It is to hope that the notorious duality of ROS/RNS action will not undermine potential therapeutic approaches as it might have already happened for some antioxidative therapies. Future attempts will have to aim at more targeted treatment strategies that take into account the adaptive side of ROS/RNS and may have to be redox modulatory rather than just simply antioxidative in nature. Further, the roles of different sources of ROS/RNS (for instance, NOX family NOXs, xanthine oxidase, and the distinct isoforms of NOS) that are not discussed in this article will merit greater attention, also with regard to their potential therapeutic exploitation.

Acknowledgments

The authors are supported by the Swiss National Science Foundation (SCORE 3232B-111352 and 3200B-111353 to G.M.K.) and project grants from the Swiss Heart Foundation (to G.M.K. and to O.P.) and the SwissLife Jubiläumsstiftung (to G.M.K.), as well as by the Mach-Gaensslen Foundation Switzerland and the Kardiovaskuläre Stiftung Basel (to O.P.).

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Date of first submission to ARS Central, July 8, 2010; date of final revised submission, August 10, 2010; date of acceptance, August 10, 2010.

Abbreviations Used

ABC = ATP-binding cassette
Cu/Zn-SOD = copper-zinc superoxide dismutase
eNOS = endothelial nitric oxide synthase
ERK = extracellular signal-regulated kinase
ET-1 = endothelin 1
GSK = glycogen synthase kinase
iNOS = inducible nitric oxide synthase
MEK = mitogen-activated protein kinase kinase
NOS = nitric oxide synthase
NOX = NADPH oxidase
PI3K = phosphoinositide 3-kinase
PTEN = phosphatase and tensin homolog
RNS = reactive nitrogen species
ROS = reactive oxygen species
SP = side population

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