Proteostasis and REDOX state in the heart

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Introduction

With over 100,000 proteins, the mammalian proteome encompasses various sizes (3–1000 amino acids), shapes (α-helices and β-sheets), functions (i.e., structural dynamic protein complex such as sarcomere, enzymes, receptors, ion channels, etc.), and neighborhoods [such distinct cellular compartments: nucleus, endoplasmic reticulum (ER), and mitochondria] in a cell. Proteome homeostasis, termed “proteostasis,” is dynamically achieved by coordinating protein synthesis (gene expression), organization/folding (with the intervention of molecular chaperones and protein-protein interactions), and degradation [calpain, ubiquitin-proteasome system (UPS), and lysosome-dependent autophagy with molecular chaperone contribution] [see reviews on proteostasis (40), sarcomere maintenance (13), and degradation-UPS (74)]. Therefore, the loss of proteostasis is among the common but not widely appreciated features of pathophysiological stresses generated by distinct cardiovascular diseases leading to cardiac dysfunction.

Reduction-oxidation (REDOX) reactions are constantly handling electron transfer from one molecule to another. This drives the energy-ATP production during aerobic respiration and oxidative phosphorylation (OXPHOS) via the electron transport chain (ECT) in mitochondria. Electrons can prematurely escape and reduce molecular oxygen to generate reactive oxygen species (ROS) (Table 1). When there is an excess of ROS production and a deficit in ROS scavenging mechanisms, cells experience deleterious oxidative stress (7). REDOX imbalance has direct effects on protein environment and proteostasis. Unfortunately, the concepts of “cellular REDOX state” or “REDOX environment” are often used without clear definitions leading to the most mistaken...
assumption that the REDOX state simply represents a global cellular characteristic (Table 1).

Like other cell types, cardiomyocytes share basic features of proteostasis and REDOX state, but they are postmitotic, highly specialized, force-generating, and beating cells (13). In terms of proteostasis, there is permanent turnover of contractile proteins in an environment under pressure to maintain the REDOX equilibrium due to the high number of mitochondria, which produce the required ATP energy while generating potentially deleterious ROS (Table 1, and Fig. 1). In fact, heart failure accompanies significant imbalances in REDOX state (oxidative/reductive stress) and alterations of numerous proteins, which are causally linked to myocardium dysfunction. Major clinical trials to test the efficacy of antioxidant treatment targeting REDOX equilibrium in heart diseases have, so far, been disappointing. Said trials question whether these therapeutic approaches are ineffective in reaching the precise sites of actions, indiscriminately modify global REDOX parameters, or promote deleterious consequences in susceptible individuals (11).

To cover the vast topic of proteostasis and REDOX state, this review is divided into five subsections:

1) Primer on protein translation, folding and degradation in cardiomyocytes;
2) REDOX state: cellular and subcellular effects on proteostasis;
3) Cytosolic/ER stress response and Protein chaperone: critical intermediates between proteostasis and REDOX state;
4) Proteostasis, REDOX state, and cardiac diseases; and
5) Therapeutic agents: cardiac friends and foes in the fight.

Table 1. REDOX-related definitions

<table>
<thead>
<tr>
<th>Terms</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Redox cycle</td>
<td>Two half reactions corresponding to oxidation (i.e., electron loss) and reduction (i.e., electron gain) for completion</td>
</tr>
<tr>
<td>Redox state</td>
<td>Ratio of the interconvertible oxidized and reduced form of a specific redox couple (e.g., NAD+/NADH)</td>
</tr>
<tr>
<td>Redox environment</td>
<td>Summation of the products in the reduction potential and reducing capacity of the linked redox couples as found in a biological fluid, organelle, cell, and tissue</td>
</tr>
<tr>
<td>Redox couple</td>
<td>A pair made of the interconvertible oxidized and reduced form</td>
</tr>
<tr>
<td>ROS</td>
<td>Superoxide, hydrogen peroxide, hydroxyl radical</td>
</tr>
<tr>
<td>RNS</td>
<td>Nitric oxide and peroxynitrite</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>Imbalance redox state with increased level of ROS and RNS</td>
</tr>
<tr>
<td>Reductive stress</td>
<td>Imbalance redox state with an increased levels of reducing equivalents in the form of redox couples (e.g., GSH/GSSG, NADPH/NADP, etc.)</td>
</tr>
</tbody>
</table>

REDOX, reduction-oxidation; ROS, reactive oxygen species; RNS, reactive nitrosative species.

-Cysteine residues, which easily accept and donate (an) electron(s), are referred as to a redox-active cysteines that are generally thiolated at physiologic pH.

Those residues can be oxidized, leading to the following modifications:

<table>
<thead>
<tr>
<th>Reversible modifications</th>
<th>Irreversible modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) disulphide bond formation : SH -&gt; S-S</td>
<td>1) sulfonic acid formation:</td>
</tr>
<tr>
<td>2) sulfenation : R-SH -&gt; R-SOH</td>
<td>R-SH + 3H₂O₂ → R-SO₂H + 3H₂O</td>
</tr>
<tr>
<td>3) nitrosylation : R-SH -&gt; R-SNO</td>
<td>2) 4-hydroxynonenal adducts:</td>
</tr>
<tr>
<td>4) glutathionylation : R-SH-&gt; R-SG</td>
<td></td>
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</table>

-Several amino acids can be irreversibly altered by oxidative attack leading to the formation of protein carbonyls (P C=O).

Their presence is the sign of higher level of oxidative stress.

<table>
<thead>
<tr>
<th>OXIDATIVE ATTACK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct</td>
</tr>
<tr>
<td>proline</td>
</tr>
<tr>
<td>arginine</td>
</tr>
<tr>
<td>threonine</td>
</tr>
<tr>
<td>lysine</td>
</tr>
<tr>
<td>cysteine</td>
</tr>
</tbody>
</table>

Fig. 1. Main oxidative protein residue post-translational modifications. Amino acids can be differently modified under oxidative environment. Those modifications can be reversible or not, induced directly or indirectly. Those posttranslational modifications are important as they affect protein structure and function.
Primer on Protein Translation, Folding, and Degradation in Cardiomyocytes

Heart tissue contains various types of cells (i.e., cardiomyocytes, fibroblasts, smooth muscle cells, and endothelial cells) among which the contractile cells represent about 30–40% in number but 75% in cellular volume (including 35% volume occupied by mitochondria) (67, 98, 108). In contrast to other cell types found in organs such as skin or intestine, the adult cardiomyocytes have very limited regenerative capacity as the cell turnover rate was recently estimated between 0.4 and 1% per year in humans (9).

For organ integrity and maintenance, cardiac cells must rely on the renewal of the cellular components, in particular the proteins that represent on average 16% of the heart weight [reviewed in (41, 87)]. Protein synthesis and degradation are tightly coupled biological processes that maintain equilibrium conditions under normal contractile activity. However, when cardiac workload increases, the level of protein synthesis is significantly augmented such that phenotypic changes of cardiomyocytes lead to physiological or pathological cardiac hypertrophy, depending on the demands for higher contractile activity (see Cardiac hypertrophy). To investigate the molecular mechanisms of cardiac hypertrophy, a classical method is the in vitro manipulation of isolated cardiomyocytes, whose size and the incorporation of leucine \(^{3}\)H can be measured as indexes of cardiac hypertrophy and protein synthesis, respectively. In response to various hypertrophic stimuli, cardiac size and protein synthesis were found to increase on average to the same extent. Conversely, reduced hemodynamic workload leads to cardiac atrophy as demonstrated in vivo by heterotrophic isografts, which can maintain perfused hearts for prolonged periods of time (54). Reduced cardiac mass was shown to result from a significant decrease in protein synthesis [up to 50% in <2 wk, (53)], as well as from elevated activity of UPS (80). Such responses suggest the existence of mechanosensors in cardiomyocytes, which can link the intensity of contraction to the molecular pathway regulating protein synthesis and/or degradation. One example illustrating such possible mechanostasis link is the muscle LIM protein, which is localized to the membrane and translocated to the nucleolus upon excessive contraction. This muscle LIM protein translocation is associated with a threefold increase in ribosomal S6 protein, which is likely to impact the level of protein synthesis (12).

More specifically, myosin and actin are particularly enriched in cardiomyocytes and form, in association with numerous other critical proteins, the cardiac contractile apparatus, organized in a structural-functional subunit, termed the “sarcomere” (13). The protein turnover rates from protein synthesis to degradation have been extensively evaluated for the cardiac contractile proteins. For example, the basal half-life can be as long as 15 days for myosin (71) or much shorter, as in the case of troponin subunits (T1/2, 3–5 days). Thus there is a large variability of stability among proteins participating to the same contractile structure, and this can have important consequences for cardiac function under pathological conditions.

In all cells, cardiomyocytes in particular, a very delicate step between the translation from the messenger RNAs and the assembly of the amino acid chain into a functioning product corresponds to the appropriate folding and organization of the amino chain in three-dimensional structure. The historical paradigm has established a strong link among protein structure, function, and well-ordered conformation. Nevertheless, cumulative bioinformatics evidence indicates that 25 to 30% of eukaryotic proteins are mostly disordered, whereas 50% of the proteins are partially disordered (96). This implies even more dynamic requirements for proteins to reach their functional status. Completion of protein folding and functional organization are not entirely based on a spontaneous process but require transient association with molecular chaperones in which productive interactions are heavily influenced by errors and other properties in the primary sequence of the protein (genetic mutation, see http://cardiogenomics.med.harvard.edu/project-detail?project_id=230#data) and REDOX environment (see next paragraph). Misfolded proteins are typically targeted for degradation. Such molecular mechanisms underlie the inherited cardiomyopathies caused by mutations in sarcomeric or sarcomere-associated proteins, whose accumulation and aggregation have dramatic consequences on cardiac function.

Cardiac proteostasis is functionally related to the assembly of the sarcomere from the sarcomeric and myofibrillar proteins, which are synthesized, folded, and further assembled in multiprotein structures through a sequential complex process called sarcomerogenesis. The most mature myofibrils are found in the perinuclear region, whereas the Z-disc corresponds to a subcellular region where defined sarcomeric compounds are assembled (13). The addition of new sarcomeres to the structure is operated within an hour, indicating a highly dynamic process that depends tightly on proper protein conformation and interaction. Finally, sarcomeric addition can occur either in parallel (concentric hypertrophy) or in series (eccentric hypertrophy) in response to different external mechanical stressors (pressure or volume overload). Altogether, these observations illustrate the different levels of protein regulation from synthesis to assembly of multi-molecular structure as the sarcomeric apparatus.

In contrast to protein synthesis that operates through a single ribosomal machinery, three distinct and complementary modus operandi have been identified for destroying cellular components and organelles: namely, the calpain/calpastatin system, the UPS, and the organelle-dependent autophagy. Several reviews have recently addressed the role played by these three different but interconnected mechanisms of cardiac sarcomere proteolysis-degradation (72, 74, 75). While those proteolytic processes are functional and required in the normal heart, they become critical in stressed hearts and, therefore, can be the object of therapeutic manipulations (see Cardiotoxicity as a severe drawback for efficient cancer drugs).

Often neglected, there is a fourth temporal dimension that, under circadian control, affects both synthesis and protein degradation processes in the heart. Such coordinated activities occur during periods of low activity when heart rate and blood pressure are reduced, creating optimal conditions for sarcomere repair and regeneration (13).

REDOX State: Cellular and Subcellular Effects on Proteostasis

Living organisms are constantly exposed to changes in their environment (nutrients, temperature, day-night cycle, exposure to pathogens, toxicants, etc.), and diverse differentiated cells have to adjust themselves to these variable conditions to
maintain REDOX homeostasis. The reader may consult the main definitions related to REDOX state in Table 1. Importantly, REDOX reactions do not reach thermodynamic equilibrium, but biological homeostasis requires a complex network of interacting REDOX couples regenerated by enzymatic pathways (33). Imbalaces of cellular REDOX homeostasis can occur when the generation of ROS exceeds existing antioxidant capacity (oxidative stress) or, conversely, when antioxidant capacity exceeds ROS generation (reductive stress). It is critical to maintain REDOX homeostasis since imbalanced REDOX causes severe damages.

The intracellular environment of cardiac cells is not a homogeneous milieu, nor is the cardiac REDOX state (84). Table 2 depicts several features of the cellular compartmentalization, including the steady-state REDOX potentials of the major REDOX couples in organelles and cytoplasm (Table 2). Cardiomyocytes exhibit a defined organization of their cytoplasm, occupied mainly by the highly structured protein complex forming the sarcomeric-contractile apparatus. Cardiomyocytes also contain organelles forming subcellular compartments with common and specific proteomes (nucleus, mitochondria, ER, and lysosomes). Those compartments display different characteristics for proteostasis, REDOX state, and regulatory proteins including chaperones (33, 45, 47) (Table 2). Although the intracellular environment is mostly reductive, there are two oxidizing compartments in the cell: namely, the mitochondria intermembrane space and the ER. Despite existing qualitative assessments of the REDOX state in intracellular compartments, the more desirable quantitative determinations, especially of REDOX potential at subcellular levels, remain difficult, challenging, and a critical unfinished task for the entire field (30, 63) (Table 3).

**Mitochondria.** The heart has a constant need for energy, and 35% of the cardiomyocyte volume is occupied by mitochondria. Mitochondria produce ATP through OXPHOS along the ECT, which includes complex I and III as two main sites of ROS production. There are three subpopulations of mitochondria in cardiomyocytes, located either under the sarcoplasmic membrane [subsarcolemmal mitochondria (SS)] or between sarcomeres [intermyofibrillar mitochondria (IMF)] and around the nucleus (perinuclear mitochondria). Based on isolation techniques, two populations of mitochondria can be separated

| Table 2. Compartmentalized REDOX potential, proteins, and main chaperones |

<table>
<thead>
<tr>
<th>Cytoplasm</th>
<th>Mitochondria</th>
<th>Endoplasmic Reticulum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Steady-state redox potential of major REDOX couples</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E&lt;sub&gt;0&lt;/sub&gt; GSSG</td>
<td>–280 mV</td>
<td>–300 mV</td>
</tr>
<tr>
<td>E&lt;sub&gt;0&lt;/sub&gt; CySS</td>
<td>–160 mV</td>
<td></td>
</tr>
<tr>
<td>E&lt;sub&gt;0&lt;/sub&gt; NADP</td>
<td>–393 mV</td>
<td>–415 mV</td>
</tr>
<tr>
<td>E&lt;sub&gt;0&lt;/sub&gt; Trx1</td>
<td>–280 mV (C)/–300 mV (N)</td>
<td>–360 mV</td>
</tr>
</tbody>
</table>

**Thiol REDOX protein/enzymes**

- GSH synthesis
  - Glutamyl-cysteine synthetase & glutamate cysteine ligase (GLC): +
  - Glutaredoxin 1 (Gsr1): +
  - Glutaredoxin 3 (Gsr3): –/+ 
  - Glutaredoxin 5 (Gsr5): + + + + + + + +
  - Nucleoredoxin (Nrx): –/+ 
  - Peroxiredoxin1 (Prx1): + + + +
  - Peroxiredoxin1 (Prx2): + + + +
  - Peroxiredoxin1 (Prx5): + + + +
  - Thioredoxin1 (Trx1): low in heart
  - Thioredoxin reductase 1: low in heart

**Chaperones (main)**

<table>
<thead>
<tr>
<th>Small Hsp (with crystallin domain)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-HspB1 (Hsp25/27): + + + +, multiple tissues</td>
</tr>
<tr>
<td>-HspB2 (MKBP): +, c/sk muscle specific</td>
</tr>
<tr>
<td>-HspB3: +, c/sk muscle specific</td>
</tr>
<tr>
<td>-HspB5 (CryAB): + + + +, c/sk muscle, lens, brain</td>
</tr>
<tr>
<td>-HspB6 (Hsp20): + +, c/sk muscle</td>
</tr>
<tr>
<td>-HspB7 (CyHsp): + + + + + + + +, c/sk muscle</td>
</tr>
<tr>
<td>-HspB8 (Hsp22, H11): + +, c/sk muscle</td>
</tr>
</tbody>
</table>

**Hsp40 DNAJ**

- DNAJA1: + + +
- DNAJB1: inducible
- DNAJB5: redox/cardiac hypertrophy

**Hsp70 family**

- HspA8 (Hsp70): + + + +
- HspA1B: + + + +, inducible

**Hsp90 family**

- HspC1 (Hsp86): + + + +
- HspC4 (Hsp84): + + + + + + + +

**Chaperonin related**

- HspE (Hsp10)
- HspD (Hsp60): + + + +

The number of (+) indicates the level of expression in heart. DCM, dilated cardiomyopathy; AF, atrial fibrillation; c/sk, cardiac/skeletal; ND, not detected.

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Table 3. Methods for proteostasis and REDOX state evaluation

<table>
<thead>
<tr>
<th>METHODS</th>
<th>BRIEF DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evaluation of free radicals</td>
<td>Spectroscopic technique that detects species which have unpaired electrons</td>
</tr>
<tr>
<td>Autofluorescence imaging</td>
<td>Measurement and interpretation of energy difference between atomic or molecular states</td>
</tr>
<tr>
<td>Endogenous NADH/NADPH (nicotinamide adenine dinucleotide) and FAD⁺⁺ (flavoproteins, FP)</td>
<td>Reduced NADH/NADPH: located in both cytoplasm and mitochondria</td>
</tr>
<tr>
<td></td>
<td>Oxidized FAD⁺⁺ located in mitochondria</td>
</tr>
<tr>
<td>REDOX state in cytoplasm/cellular compartments using reporter protein (based on reactive thiol)</td>
<td>Redox-sensitive GFP: GFP with artificially inserted dithiol-disulphide pair with/without organelle-targeting signals</td>
</tr>
<tr>
<td></td>
<td>Ratiometric fluorescent measurement: ratio excitation 490/425nm = Fox/Fred</td>
</tr>
<tr>
<td>REDOX state in cytoplasm &amp; cellular compartments using probes</td>
<td>One of most commonly used: - H2DCFDA: cell permeant, not fluorescent reduced fluorescein</td>
</tr>
<tr>
<td></td>
<td>H2DCFDAdeacetylated H2DCF in cells becomes fluorescent (emission 488nm) after oxidation by ROS</td>
</tr>
<tr>
<td>Biologic thiol and disulphide REDOX state</td>
<td>NB: their specificity has been questioned.</td>
</tr>
<tr>
<td>GSH/GSSG from cell extracts</td>
<td>-HPLC -enzymatic /colorimetric assay</td>
</tr>
<tr>
<td></td>
<td>- GSH + DTNB (5,5'-dithio-bis-2-nitrobenzoic acid) = TNB (yellow colored 5-thio-2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>GSH/GSSG in living cells using reporter proteins</td>
<td>Grx1-roGFP2: fusion of roGFP with glutaredoxin</td>
</tr>
<tr>
<td></td>
<td>Ratiometric fluorescent Measurement: ratio 408/488nm = ↑ with oxidation</td>
</tr>
<tr>
<td>SH-proteins/S-S-proteins</td>
<td>- Redox Western Blot</td>
</tr>
<tr>
<td></td>
<td>- ICAT (Isotope Coded Affinity Tags)</td>
</tr>
<tr>
<td></td>
<td>- SH-proteins + AMS = MW ↑</td>
</tr>
<tr>
<td></td>
<td>- SH + 12C = 12C-S</td>
</tr>
<tr>
<td></td>
<td>- S-S + TCEP + 13C = 13C-S</td>
</tr>
<tr>
<td></td>
<td>quantitative LC/MS</td>
</tr>
<tr>
<td></td>
<td>(12C-S/13C-S)</td>
</tr>
<tr>
<td></td>
<td>C-proteins DNPH (2,4-dinitrophenylhydrazine) derivatized protein</td>
</tr>
<tr>
<td>Carboxylation detection</td>
<td>Western Blot probed with anti DNPH antibodies</td>
</tr>
</tbody>
</table>

(non-IMF or SS and IMF) and shown to be morphologically and biochemically different (49, 82). Such fractions were shown to have different levels of activities for mitochondrial complexes of the ECT and to be differentially affected by oxidative posttranslational modifications of OXPHOS proteins (70). For example, protein carbonylation affects more severely SS mitochondrial proteins, whereas nitration is found higher in IMF organelles. ATP synthase subunits are among the most carbonylated proteins in both types of mitochondria. This can impact REDOX state as mitochondrial ECT dysfunction often leads to oxidative stress.

Such oxidative modification of proteins can impact other mitochondrial functions such as the control of apoptosis (49) or the cardiac Ca²⁺− signaling (25, 60). The IMF mitochondria are surrounded by a network of membranous compartments forming sarcoplasmic reticulum and are positioned close to microdomains of elevated local Ca²⁺−. Evidence for physical coupling between the outer mitochondrial membrane and the sarcoplasmic reticulum has led to the speculation that a potential cross talk could also exist at a REDOX-state level between the two compartments (26).

These interactions remain to be further investigated, in particular to determine whether there is any further correlation between SS and IMF mitochondria with the mitochondrial specific REDOX couples and antioxidant enzymes: thioredoxin2/thioredoxin reductase 2, MnSOD, and nicotinamide adenine dinucleotide (phosphate), which produces NADPH essential to maintain GSH/GSSG (Table 2).

Although endowed with its own genome and protein synthesis apparatus, mitochondria are dependent on the nuclear genome for synthesis of numerous nuclear-encoded proteins subsequently imported in the organelle. Similarly, mitochondria rely on the synthesized/recycled pool of GSH produced in the cytoplasm since they can only recycle it. GSH enters the organelle using carboxylate or oxoglutarate carriers (8, 59). This is another indication of interactions between cellular compartments, and despite its potential critical importance for REDOX balance of the mitochondria, this mechanism of import has surprisingly received little attention to date.

Endoplasmic reticulum. In cardiac cells, the ER compartment exerts multiple functions related to protein folding and maturation of membrane-bound and secretory proteins, N-linked protein glycosylation, phospholipid, and steroid synthesis. It is also critically involved in Ca²⁺− dynamic storage with ryanodine receptor, releasing the ion in the cytoplasm while sarco(endo)plasmic reticulum Ca²⁺−-ATPase pumps it back into the ER compartment during each diastole (35). Cardiac ER is also organized in various domains with specific properties
regarding Ca\(^{2+}\) handling (sublocalization of the Ca\(^{2+}\) channels) and interactions with mitochondria (25). Regarding protein folding, the ER compartment is a well-known, highly oxidizing environment (61). New proteins entering the ER compartment upon translation and translocation are constantly introducing reducing equivalents. To maintain such an oxidizing REDOX environment, ER contains specific protein such as ER oxidoreductin (ERO) and ERO1α and -β, the two isoforms existing in mammals. Those proteins function through an ERO-protein-disulfide isomerase (PDI) pathway where reactive cysteines located on both proteins play an important role, regulating each other’s activity (61). ERO1 activity can generate ROS, which can diffuse across the ER membrane, potentially leading to oxidative damages of ER intramembranous and/or cytoplasmic proteins. Because mouse models deficient in both ERO1 isoforms exhibited minor phenotype, it was suspected that this molecular machinery was working in parallel with some other REDOX proteins. Peroxiredoxin 4 was identified as one efficient candidate able to oxidize PDI (112).

It is worth mentioning that none of these REDOX proteins is highly expressed in the heart (ERO1α and -β, PDI, and peroxiredoxin 4) in contrast to actively secreting cells. Nevertheless, peroxiredoxin 4 exhibits a defined profile of expression in the heart with a higher expression in the atria than in the ventricles and a higher expression in the right ventricle than in the left ventricle (atria > right ventricle > left ventricle).

There is a constant and complex communication between the more reduced cytoplasm and the highly oxidized ER. Reducing equivalents can enter the ER via transmembrane electron carriers [NADPH oxidase 4 (NOX4), cytochrome B, and lipo-philic vitamins] or through specific transporters which import GSH and other protein thiols. Oxidizing equivalents are also collected from the cytoplasm to ER lumen (e.g., FAD) (61).

Cytosolic/ER Stress Response and Protein Chaperone: Critical Intermediates Between Proteostasis and REDOX State

Since the heart has special energetic and protein regeneration-maintenance requirements, one could expect a high demand for molecular chaperones to secure cardiac proteostasis. Chaperones transiently bind with other proteins (clients) to assist their proper folding (or refolding) so that client proteins achieve their proper conformation and functional competency (8, 21, 32, 47, 102). Chaperones are found in the cytoplasm as well as in the different cellular compartments such as ER or mitochondria (Table 2).

Heat shock proteins (HSPs) are the best-known chaperones, and their expression in the heart versus other tissues can be compared using available microarray data (BioGPS) (104) (Table 2). Several HSPs belonging to the different families, mainly organized based on their molecular weight, are more expressed in the heart than in other tissues (47). In particular, the small HSPs are well represented among abundant HSPs in the heart: HspB1 (Hsp25/27), HspB2 (myotonic dystrophy protein kinase-binding protein), HspB3, HspB5 (α-β-crystalline), HspB6, HspB7, and HspB8 [reviewed in (93, 99)]. Despite the tissue-specific enrichment of HspB1, HspB2, and HspB5 during development and in the adult heart, the single deficiency in HspB1 or the combined deficiency for HspB2 and HspB5 did not severely modify the cardiac phenotype in knockout mice (14, 44). This could be explained by a potential compensatory mechanism between the numerous small HSPs expressed in heart. While there is not yet any knockout line for the other members of the small HSPs family, multiple experiments demonstrate their importance in cardiac function under pathological stress [e.g., HspB6 (28) and small HSPs (26, 27, 50, 56)].

In addition to the physiological expression of chaperones, stress conditions can upregulate several of them as a defense mechanism (heat shock response and ER stress response) (64, 102) (Fig. 2). One of the stress conditions existing in cardiomyocytes corresponds to important changes in REDOX state, which impact protein conformation, leading to protein misfolding and dysfunction in the different cellular compartments. Following deleterious REDOX state modifications, heat shock factor 1 (HSF1), the master regulator of heat shock response, is concurrently activated by its own change in conformation (5) and is released from a multichaperone complex, which preferentially interacts with abnormal protein. Activated HSF1 translocates into the nucleus and induces the expression of numerous chaperones or HSPs in addition to other genes participating to the stress response (94). Abnormal proteins can also accumulate in the ER, inducing the ER stress response. This response includes three different transcriptional regulators [X-box binding protein 1 (XBP1) and activating factor 4 and 6 (ATF4 and -6)], which are activated and induce chaperone expression, in addition to mediators of other cellular mechanisms such as apoptosis (64).

Proteostasis, REDOX State, and Cardiac Diseases

Cardiac dysfunction of various etiologies (i.e., ischemia, and arrhythmia) exhibits global and, perhaps, localized changes in REDOX imbalance, either oxidative or reductive stress. Multiple deleterious consequences of such imbalance impact 1) the regulatory pathways based on ROS signaling; 2) the expression and/or function of proteins involved in the dynamic Ca\(^{2+}\) homeostasis; and 3) the myofibrillar proteins, among others (84, 91). In particular, REDOX-dependent posttranslational modifications (e.g., disulfide bond and carboxylation) alter the conformation of several myofibrillar proteins (Fig. 3), and this is likely to provoke functional changes of the sarcomeric contractile apparatus. Additionally, in the case of oxidative stress, specific subunits from the 19S proteasome become oxidized, leading to a significant reduction of the 26S proteasome activity (23). Likewise, elevated levels of ROS stimulate autophagy that, in turn, paradoxically lowers ROS by still poorly defined mechanisms [e.g., experimental work described in (107); and reviewed in (37)]. Shifts in the equilibrium between protein synthesis and degradation are guaranteed to alter the maintenance of cardiomyocyte homeostasis. Without a detailed understanding of the mechanisms involved (both deleterious and protective), therapeutic approaches that aim to reduce the harmful consequences on molecular defenses which can reverse cardiac dysfunction are likely to be stymied.

Myocardial ischemia. Ischemia-reperfusion (I/R) injury caused by a severe disruption in oxygen and nutrient supply dramatically impacts mitochondrial function and NOX activity. From data acquired by electron paramagnetic resonance microscopy (Table 3), the release of reactive oxygen-centered free radicals and burst of oxygen radical generation occur within moments...
of reperfusion. In isolated hearts, ischemic hearts after 10 min of ischemia and 10 s of oxygenated reperfusion led to a sixfold increased signal compared with that in control samples (115).

Significant bursts of oxidative stress are initial targets for the antioxidant enzymatic (e.g., superoxide dismutase) and nonenzymatic pathways (e.g., glutathione pathways). In addition, the recruitment of regulatory defense systems against the deleterious effects of I/R occurs under the control of several redox-sensitive transcription factors such as hypoxia-inducible factor-1α (HIF-1α), HSF1, Nrf-κB, and p45 NF-E2-related transcription factor (NRF2).

Extensive studies have established that the regulatory mechanisms governing HIF-1α pathway by ischemia are mediated at the levels of transcriptional, translational, and degradation (85). Discovered initially as the central regulatory mechanism for the upregulation of erythropoietin gene expression, HIF-1α constitutes an evolutionarily conserved oxygen-sensing mechanism responsible for scores of genes and physiological processes from angiogenesis to glycolysis to hypoxia. HIF-1α forms a heterodimeric complex of HIF-1α and HIF-1β subunits with properties of heterodimerization and DNA-binding activity but with dramatically distinct half-life of protein stability. Under normal conditions, HIF-1β remains constitutively expressed, whereas HIF-1α has a half-life of <1 min when sequential interactions with proline hydroxylases (PHDs) and von Hippel-Lindau tumor suppressor trigger rapid degradation by the proteasome (65).

In response to hypoxic stimulus, the activity of oxygen-sensor PHDs is significantly reduced and HIF-1α remains unhydroxylated, preventing interactions with von Hippel-Lindau tumor suppressor and promoting its stabilization. Therefore, the degradation of HIF-1α decreases because of the reduced activity of PHD due to conformational changes with low oxygen and higher ROS but also because of proteasome lower activity. During acute myocardial ischemia, HIF-1α activation is cardioprotective because of its regulatory targets of genes encoding Ca2+ handling and metabolic and angiogenic pathways. There are also interactions between HIF-1α and other factors such as HSF1 (6, 114). However, unregulated HIF-1α activation can be also deleterious, as recently shown by persistent stimulation of HIF1 activity, which causes cardiomyopathy (66).

In contrast to HIF-1, the expression of HSF1 is typically unchanged in response to an acute stress. HSF1 activation is regulated by posttranslational modifications, REDOX-dependent changes in conformation, and the release from inhibitory interactions involving HSPs (Fig. 2). Of interest, CAMKII δ B was recently suggested to link oxidative stress and HSF1 activation in neonatal rat cardiomyocytes. With the increase of the level of S230 phosphorylation on HSF1, CAMKII δ B was significant...
shown to augment stress-inducible HSP70 expression (73). That activated HSF1 exerts beneficial roles during I/R was demonstrated using a transgenic mouse model expressing a constitutive active form of HSF1 (human β-actin-ΔHSF1) (113). Carboxyl-interacting protein of HSP70 (CHIP) normally contributes to HSF1 activation, but CHIP null mice exhibited a much more severe phenotype following experimental I/R (109). As expected, HSP expression correlated with HSF1 activity. In hearts of human β-actin-ΔHSF1 mice, the HSPs level was doubled, and beyond their protective role as chaperones, HSPs were shown to stimulate prosurvival pathway as exemplified by phosphorylation of Akt and to lower the pro-death pathway conducted by phosphorylated form of JNK. Several transgenic mouse models were generated to allow the overexpression of various HSPs, either directly regulated by HSF1 (HspB1, HspB5, and HspA1A) (18) or not (HspB6) (28, 29). Taken together, there is now compelling evidence that increased proteostasis mechanism(s) provide robust protection under I/R-induced REDOX imbalances.

Another important major regulator pathway for sensing oxidative stress cellular environment in the heart is NRF2 [reviewed in (69)]. NRF2 is a REDOX-sensitive transcription factor that is first negatively regulated by an interaction with Kelch-like ECH-associated protein-1 (Keap1), an E3 ligase responsible for its ubiquitination and degradation. Upon oxidative stress, Keap1 cysteine residues (Cys151) serve as stress sensors such that conformation changes of Keap1 prevent its interaction that promotes degradation of NRF2. In addition, NRF2 can be phosphorylated by ER stress mediator, ER stress-activated eIF2α kinase (PERK), contributing to its dissociation from Keap1 (19). Then NRF2 migrates into the nucleus to achieve DNA-binding activity of downstream targets. NRF2 protein contains critical cysteine that needs to be maintained in the reduced form, and this is achieved via REDOX reactions with the thiol-containing GSH and, more importantly, thioredoxin systems. NRF2 binds specific DNA sites described as antioxidant responsive element found in the regulatory regions of many genes such as NAD(P)H quinone oxidoreductase, gamma glutamylcysteine (GSH synthesis), glutathione-S-transferase, and heme oxygenase, which is also regulated by HIF-1 and HSF1. Several studies place NRF2 as a key transcription factor interacting with other REDOX regulators such redox effector factor-1, NF-κB (36), and NOX4, whose protective activity following I/R would be entirely mediated by NRF2 (15). NRF2 signaling mechanisms are being actively investigated since the range of downstream effectors could be therapeutically targeted for boosting antioxidative defenses.

Because ischemia generates ROS and protein oxidation, the molecular and cellular mechanisms for eliminating those abnormal proteins are critical for the survival of the affected cardiomyocytes in the ischemic heart. Proteasome activity is involved in eliminating oxidized actin (22, 75). Another protective mechanism, potentially under the proteasome control, is the degradation of phospho-c-jun implicated in triggering apoptosis (58). Ischemia also stimulates autophagy. Transgenic green fluorescent protein-microtubule-associated protein-1 light chain-3α (GFP-LC3) mice are an interesting model to monitor the autophagic activity since LC3 is specifically associated with autophagosomes. Thus the activation of autophagy after ischemia is revealed by the formation of GFP-LC3 aggregates, and this phenomenon is particularly visible in rescued cardiomyocytes located in the ischemic border zone region (48).

In this review, the focus has been restricted on the cardiomyocytes, but they also interact with their cellular environment which can provide various signals including changes in REDOX environment, such as communication between endothelial cells and cardiomyocytes [e.g., activation of neuregulin/
Cardiac hypertrophy. Cardiac hypertrophy, or an increase in cardiomyocyte mass, has many triggers (e.g., pressure/volume overload) involving cellular responses to various (patho)physiological and pharmacological signals that are integrated through complex signaling pathways (1, 24, 42). During increased contractile demand, the heart undergoes hypertrophic response mediated through dynamic changes in the equilibrium between protein synthesis and degradation, allowing sarcomere addition. In fact, there are complex links between the distinct origins of cardiac hypertrophy and the different modes of protein degradation [e.g., inhibiting calpain activity disrupts cardiac hypertrophy (72)]. This also impacts the production of energy required to cope with such an elevated workload and is likely associated with a high risk of increased ROS generation by mitochondria. At the molecular level, it was shown that induced NOX4 activity following hypertrophic signaling (angiotensin II) contributed to the elevated production of ROS by mitochondria (3). Further discussion about the intervention of NOX4 in cardiac hypertrophy and other cardiovascular diseases can be found in recent reviews (4, 88).

Manifestations of both abnormal proteostasis and REDOX state imbalances were shown in a mouse model, recapitulating a human desmin-related cardiomyopathy due to the R120G mutation in the α-B-crystallin gene (HspB5, Table 2). Although R120G mice express the mutated HSPB5 along with the endogenous wild-type protein, by the age of 6 mo they show large aggregates occupying nearly the entire cardiomyocyte and severely disrupting the sarcomere organization (62). In addition, cardiomyocyte REDOX state is profoundly perturbed with an increased level of reduced GSH, leading to reductive stress. Glucose-6-phosphate dehydrogenase (G6PD) enzymatic activity is elevated in the mutant cardiomyocyte, and this can contribute to the observed imbalanced REDOX state since this enzyme plays a key role in the pathway producing NADPH, which is an essential cofactor of glutathione reductase in its catalysis of oxidized to reduced glutathione. Since significant cardiac hypertrophy with a high level of atrial and brain natriuretic factor expression is found in 6-month old R120G male mice, this suggested a causal link between G6PD activity and cardiac hypertrophy. This hypothesis was supported by the beneficial effect of introducing the R120G mutation in a G6PD-deficient genetic background (77). A second line of evidence is brought by a gain of function experiment overexpressing HspB1. Transgenic mice with a very high level of HSPB1 have a higher level of GSH and develop cardiac hypertrophy (110). How HSPB1 can lead to an increased GSH amount can be explained with previous work by Arrigo and colleagues (76), who had shown that HSPB1 is able to interact with G6PD and stimulate its activity. The link between R120G expression and reductive stress is likely to be complex, relying on multiple REDOX regulators. This is illustrated by experiments showing that the antioxidant factor, NRF2, is activated in R120G transgenic mice with the expected increased expression of NRF2 target genes [e.g., NAD(P)H quinone oxidoreductase and catalase] and a possible contribution to the REDOX imbalance in favor of higher antioxidants and reduced milieu (78).

Downstream the hypertrophic signaling pathways, a complex transcriptional regulation involving epigenetic modifications regulates the expression of hypertrophic genes [e.g., histone deacetylase (HDAC) (38, 51)]. This step further associates proteostasis and REDOX state as illustrated by the work performed by the laboratory of Sadoshima and colleagues (2). They demonstrated that HDAC4, a member of the class II HDAC, is regulated by REDOX reactive cysteines, Cys^{667} and Cys^{669} (2). DNAJB5, of which the level of expression is increased by thioredoxin 1, forms the REDOX-sensitive intermediate between thioredoxin1 and HDAC4 so that HDAC4 reactive cysteines remain reduced. This prevents HDAC4 export to the cytoplasm. SH-HDAC4, as it is located in the nucleus, ultimately maintains hypertrophic genes under a repressive-underacetylated chromatin.

Finally, as described in the context of I/R, cardiac hypertrophy is also associated with the cellular and ER stress responses mediated by the previously mentioned transcriptional factors (in particular HIF1-α, HSF1, and NRF2). Some level of interactions or cross talk between those different transcription factors is expected as their actions should converge to protect the cardiac cells. This was recently illustrated by the work performed with HSF1 mouse models. Zhou and coworkers (114) demonstrated that HSF1 and HIF1-α are functionally connected to increase the expression of VEGF and stimulate beneficial angiogenesis after experimentally induced pressure overload.

Arrhythmia: atrial fibrillation. Atrial fibrillation (AF) is among the most common cardiac dysrhythmias. It has a lifelong risk for development in one of every four men and women over 40 years of age (59). Since AF imposes rapid and uncoordinated contraction on cardiomyocytes, the duration of the arrhythmia is an important factor inducing atrial remodeling (both electrical and structural) but also the activation of calpain, a sign of unbalanced proteostasis. In addition, AF leads to Ca^{2+} overload and this has been shown to be further associated with oxidative stress. In fact, accumulating evidence has supported the hypothesis of AF and oxidative stress as summarized by Huang and colleagues (43). This includes histological studies, which demonstrate the oxidative damage in case of AF, the presence of markers of oxidative stress (nearly quadrupled protein carbonylation; diminished level of reduced SH) (16) and some beneficial effects of antioxidant treatment.

As a link between proteostasis and REDOX state, the expression of HSPs, in particular HSP70 and HspB1 (Hsp25/27), is modified in chronically affected patients (46). In tachycardia-paced HL-1 atrial myocytes, an experimental model of AF, HspB1 and other members of the small HSP family were found to exert beneficial effects through distinct mechanisms. HspB1, -6, -7, and -8 were able to mitigate the reduced level of Ca^{2+} transients. HspB1, -6, and -7 intervene at the level of structural remodeling of actin stress fibers, and HspB8 specifically acts on RhoA GTPase, an upstream regulator of the cytoskeleton reorganization (50). In those studies, the REDOX state was not analyzed, so beyond HspB1, it remains to be determined whether the other small HSPs highly expressed in the heart can rescue an imbalanced REDOX state.

Therapeutic Agents: Friends and Foes in the Fight

Cardiovascular diseases represent one of the leading causes of morbidity and mortality in Western countries. Substantial insights on the basic mechanisms, etiologies, diagnostics, and
therapeutics have fundamentally changed the management of cardiovascular diseases in recent decades. Therapeutic intervention interfering with REDOX state can have both positive and negative effects, as exemplified in the following selected examples.

**Hype and hope in search for cardioprotective drugs.** As mentioned elsewhere (52), the preponderance of evidence of oxidative stress in most cardiac pathological processes has logically justified interventions using antioxidant drugs and dietary supplements (e.g., vitamins A, E, and C) for disease prevention. However, the positive results of antioxidants in experimental studies have not been confirmed in clinical studies that have even established increased adverse outcomes and toxicity of antioxidants (39). In the Medical Research Council/British Heart Foundation Heart Protection Study, for example, there were no significant differences between 20,536 individuals randomized to either supplementation with antioxidant vitamins or normal diet in the number of nonfatal myocardial infarction or coronary deaths [cited from (39)]. Likewise, among 58,730 Japanese individuals, there was no significant difference for men with vitamin C intake, but an inverse association for increased mortality from cardiovascular disease was found among Japanese women (55).

A substantial literature has documented the improvements of cardiac pathologies linked to oxidative stress by overexpression of HSP chaperones, providing proof of concept for beneficial targets of proteostasis boundaries [e.g., protective effect of HSP overexpression in transgenic mice (18)]. This concept awaits further translational studies from the bench to bedside and has yet to further prove the benefits of drugs with such capability of inducing chaperones.

Geranylgeranylacetone (GGA), an acyclic polyprenoid and antiulcer drug developed in Japan, was first tested about 10 years ago in experimental studies on I/R in rats (68). A single-dose regimen of GGA doubled the levels of Hsp70 protein expression but did not change the expression of Hsp25/HspB1, Hsp60, and thioredoxin. A positive effect was observed after GGA treatment and was thus explained by the greater amount of Hsp70. This effect on Hsp70 expression could be due to the fact that GGA activates HSF1 (92). Sanbe and coworkers (83) have recently demonstrated that long-term GGA administration stimulates the expression of two small HSPs, i.e., HspB1 and HspB8, and can suppress the R120G-related cardiac disease in animal models. This shows that the same compound can induce diverse changes in protein expression (depending on the cellular or treatment conditions) and acts through multiple molecular mechanisms, including activation of various transcription factors since HSF1 does not regulate HspB8. To date, there are no clinical reports that have directly tested whether GGA has similar benefits for distinct forms of heart disease in humans.

Celastrin is a triterpenoid compound isolated from the plant family Celastraceae. This compound was shown to induce HSF1 activity as the classical heat shock (100). While best known for its anti-inflammatory properties in part through the inhibition of NF-κB pathway, celastrin exerts several salutary effects by abrogating the deleterious consequences of oxidative stress. Celastrin was successfully used to mitigate the consequences of the hypertension-induced inflammation and oxidative stress in vascular smooth muscle. Heme oxygenase was identified as a key component without determining the molecular link between the drug and this gene, which could be some REDOX-sensitive transcription factors, either NRF2 or HSF1 (54, 86). Interestingly, in the study by Yu et al. (106), they showed that cardiac hypertrophy was reduced in celastrin-treated hypertensive rats. This observation would require more investigation to determine whether celastrin acts directly on the cardiomyocyte.

Resveratrol, a small polyphenol (3,4′,5-trihydroxystilbene) found in skin of red grapes, has generated considerable interest as a therapeutic agent in both experimental and clinical settings. Resveratrol is a potentially powerful antioxidant that can modify the expression of small HSPs in the heart (10), but its mechanisms of action remain to be better elucidated. Wu and Hsieh (104a) have recently reviewed resveratrol effects, mostly in the vascular compartment, but as already mentioned, evidence for direct impact on cardiomyocytes is accumulating. Resveratrol attenuates the cardiac toxicity of the antitumor drug, azidothymidine, by reducing the generation of mitochondrial ROS in primary human cardiomyocytes (31). A recent review summarizes the still-in-progress human clinical trials that test the efficacy of resveratrol effects for cardiovascular and other diseases (89).

**Cardiotoxicity as a severe drawback for efficient cancer drugs.** Deleterious side effects remain another challenge for new drug discovery. Significant numbers of potential drug candidates are withdrawn from the market or are eliminated from further clinical studies because of cardiotoxicity. From a better understanding of the detailed mechanisms, there is hope that either such deleterious effects might be eliminated or more appropriate pharmacological treatment can be developed. Two common and efficient cancer drugs, which are implicated in REDOX state and proteostasis, best illustrate this conundrum.

Doxorubicin (Doxo), a member of the anthracycline derivative family, is among the most widely used and effective anti-cancer drugs. Unfortunately, Doxo treatment induces irreversible cardiotoxicity and dilated cardiomyopathy in a dose-dependent manner, and extensive studies have been undertaken to identify the causal mechanisms. In parallel with the drug’s metabolism into an unstable semiquinone intermediate, Doxo administration both increases oxidative stress and induces chaperone-like HSP expression. ROS-dependent protein alterations might explain the cascade of adverse events causing cardiac dysfunction (dysregulation of Ca2+ handling) and cell death (mitochondria-dependent apoptosis) [review in (111)]. Although HSF1-dependent upregulation of HSPs contributed to the protective mechanisms for proteostasis against toxicity in cellular models [immortalized cardiac H9c2 (95)], experimental work with mouse models paradoxically revealed some opposite effects. Proteomic analysis showed an accumulation and aggregation of HSPB1 in Doxo-treated animals with heart failure. The loss of function of HSF1 reduces HSPB1 expression and consequently the HSPB1-dependent p53 induced apoptosis (97). The apparent contradiction between the results obtained by the same laboratory emphasizes the importance of considering the cell specificity of proteostasis and REDOX state. More detailed proteomics analysis confirms that Doxo treatment induces severe alterations in the mitochondria, along with antioxidant defense pathways (90). In addition, Doxo seems to activate the proteolytic machinery, in particular UPS, in such a way that critical cardiac transcription factors and survival factors are overtly degraded [review in (79)]. Based on experiments using...
animal models, the stimulating overexpression of the cardiac HSP20 (small HSPB6) could be beneficial (29).

Bortezomib (Velcade), an inhibitor of the 26S proteasome, is commonly used for treatment of multiple myeloma and other refractory lymphomas. The incidence of cardiotoxicity, including heart failure, was 15% among a group of 699 treated patients with multiple myeloma (81). Some severe, reversible cardiac failure after bortezomib treatment, combined with chemotherapy, has been reported in a case of non-small cell lung cancer (105). The mechanism of action of such a drug depends on the selective degradation of proapoptotic factors in relation to the programmed cell death in neoplastic compared with normal cells. How those drugs induce cardiotoxicity remain enigmatic but likely depend on the inhibition of the proteasome itself, in association with some age-dependent cardiac susceptibility. Recent investigations have implicated the mitochondrion, a very sensitive organelle, in the toxicity mechanism. It is tempting to speculate that mitochondria dysfunction causes oxidative stress and further alterations of the UPS, a central mechanism for removing damaged proteins to enhancing proteostasis.

Perspectives

As cited in this paper, numerous reviews have recently covered some aspects of the present topic: proteostasis and REDOX state (84, 91, 101–103). There is a considerable urgency to establish new therapies and preventative strategies to circumscribe cardiovascular dysfunction associated with protein alterations and REDOX imbalance. Additional evidence for such a need includes the demonstration of the burgeoning increases in metabolic diseases such as diabetes, altering REDOX balance, and the aging-dependent mechanisms that further reduce proteostasis capability.

In the face of this situation, intense efforts need to be devoted to improve the clinical applications of the combined uses of radiotracer and REDOX-sensitive dyes, which could herald new diagnostics with greater sensitivity and specificity of REDOX state. Furthermore, a better understanding of the complex regulation of cellular REDOX balance could facilitate the development of newer antioxidants aimed at specific cellular targets such as organelles, vessels, and cardiomyocytes (20). Certainly, previous unsuccessful clinical trials should not prevent efforts to design and deploy new innovative drug strategy testing after an adequate identification of the targets and a more efficient screening for highly specific targets in the era of personalized medicine.

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REFERENCES


PROTEIN HANDLING IN HEART


