NRF2: A CANDIDATE THERAPEUTIC TARGET TO DAMPEN OXIDATIVE STRESS IN ACUTE MYOCARDIAL INFARCTION

by

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Abstract
This literature review posits that the transcription factor Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is an attractive candidate therapeutic target in the setting of acute myocardial infarction (AMI). This transcription factor binds to antioxidant response elements (ARE) in the promoter region of a battery of genes that collectively encode an array of antioxidant, phase II drug metabolism, metabolically stabilizing, and overall cytoprotective enzymes, facilitating their transcription at basal levels and increasing transcription in response to various cellular stressors. Following a brief background tutorial on normal cardiac myocyte cellular physiology, key events that occur early in ischemia and reperfusion are outlined and integrated. These include ionic and metabolic dysregulation, electron transport chain uncoupling, mitochondrial depolarization, and the generation of reactive oxygen species (ROS). Abrupt changes in response to ischemia prime opening of the mitochondrial permeability transition pore (MPTP) and cardiac myocytes to generate a burst of ROS upon reperfusion – two key events that contribute to the umbrella term ischemia-reperfusion injury (IRI). How ROS damage cells is then outlined, and through a ROS-centric viewpoint, a case will be made as to how exogenous upregulation of Nrf2 could protect and/or salvage at-risk tissue immediately subjected to infarction and neighboring tissue in the peri-infarct zone (PIZ). The history of how Nrf2 came to be known as the “master regulator of oxidative stress” is reviewed, as well as the discovery of the canonical mechanism of Nrf2 regulation via Kelch-like ECH-associated protein 1 (Keap1) and other alternative mechanisms of endogenous Nrf2 regulation. Finally, compiling interdisciplinary evidence from research publications around the world, the benefits of therapeutically targeting Nrf2 are considered given the timescale and context of acute MI. Drug delivery methods, potential challenges, and limitations are then considered. Cardiac tissue is a dynamic substrate that exhibits changes for up to 90 days after AMI and patient outcomes are directly related to the extent of tissue lost following infarction/reperfusion. Targeting Nrf2 addresses an unmet need, as current clinical therapies focus on precluding occlusions and prompt reperfusion of infarcted tissue, but do not explicitly target at-risk tissue following infarcts and/or present-day reperfusion methodologies.

Introduction

Ischemic heart disease is the leading cause of death in the world. In addition to the millions of patients and their support systems that are directly affected, cardiovascular disease is a tremendous burden from both a public health and economic standpoint; in 2007, it accounted for 15% of total healthcare expenditures (286 billion USD) -- the most of any diagnostic classification. Despite steady decreases in smoking and an increased understanding of pathogenesis and risk factors of cardiovascular disease, there is an upward trend of sedentary lifestyles, poor diet, obesity, and diabetes in America, all of which are risk factors for and contributors toward ischemic heart disease. Major coronary events, specifically acute myocardial infarctions (MI) (both new and secondary events), affect over one million Americans each year, occurring every
34 seconds in America alone.\textsuperscript{3} The advent of pharmacological medications such as antiplatelets, anticoagulants, and statin drugs along with ongoing optimization of percutaneous intervention (PCI) and coronary artery bypass graft (CABG) methodologies has greatly improved the survival rate for those who experience acute MI due to prompt reperfusion, which limits initial infarct size. Yet while widespread access and timely implementation of the aforementioned medical and surgical therapies has led to higher survival rates, problems loom for survivors and healthcare providers; patients who live through an acute MI are at higher risk of developing heart failure, having a subsequent MI, and have a higher rate of all-cause mortality.\textsuperscript{4,5,6}

Predicting how patients will fare following acute MI is presently done in part by assessing the extent of damage to the cardiac tissue, as symptoms manifest from structural and functional changes in the cardiac tissue and cells. The degree of damage i.e. cardiac tissue death and subsequent fibrosis and/or cardiac dysfunction can be gauged directly with magnetic resonance imaging (MRI) as well as by proxy, measuring physical ability as well as mechanical and electrical heart function using stress tests, echocardiograms, or electrocardiograms, respectively.\textsuperscript{7} This dynamic tissue exhibits structural, functional, and histopathological changes for up to 90 days post-MI in animal models and in humans.\textsuperscript{8,9,10} In addition to the initial insult of ischemia, cardiac myocytes are subjected to stress upon reperfusion, deemed ischemia-reperfusion injury (IRI) and experience latent waves of oxidative stress and inflammation. A 2007 review in the New England Journal of Medicine estimated that reperfusion injury accounts for 40-50\% of the final MI size.\textsuperscript{11} The present review elaborates on the underlying mechanisms of ischemia and reperfusion injury, keeping in mind the timescale of events. This review also aims to consider the peri-infarct zone as a critical target tissue in myocardial infarction. As key cellular pathways and mechanisms of myocardial cell stress and death involved in ischemia, reperfusion, and healing phases of MI are described, one transcription factor will continuously appear as a protective entity and attractive therapeutic target: Nuclear factor (erythroid-derived 2)-like 2 (Nrf2).\textsuperscript{11}

This review will outline in detail key intracellular changes that occur as a result of abrupt ischemia as well as how these changes set the stage for widespread metabolic and ionic dysregulation and a massive burst of ROS upon reperfusion. The connection between calcium, a key ion in cardiac physiology, and ROS will also be elucidated, as dysregulation of either one potentiates dysregulation of the other and this interplay is the basis for a “bioenergetics catastrophe” that occurs during ischemia-reperfusion.\textsuperscript{166} The transcription factor Nrf2, the “master regulator of oxidative stress,” induces expression of a wide variety of gene products that protect against ROS-mediated damage and cell death. This review takes a ROS-centric view of acute MI and posits therapeutically upregulating Nrf2 levels and thereby activity and downstream gene products offers a break from the vicious cycle that occurs during ischemia-reperfusion.
The Global Myocardial Infarction Task Force defined their third iteration of myocardial infarction in 2012, which is dependent on the (high-sensitivity) presence of cardiac troponin in addition to the presence of at least one symptomatic, imaging, or electrocardiographic finding indicating MI.\textsuperscript{12} In this review, the discussion of myocardial infarction will be limited to an acute MI caused by occlusion of an epicardial artery, which results in vascular insufficiency and subsequent necrosis that begins in the subendocardium and exhibits a ‘wavefront’ of cell death over 24 hours toward the epicardium, resulting in a tapered, wedge-shaped scar months later.\textsuperscript{13} That is not to say that the timeline of cell death and cases made to explore Nrf2 as a novel therapeutic target to salvage cardiac tissue are inapplicable to cases of global ischemia secondary to cardiac arrest or forms of hypotensive shock. It is simply not feasible to discuss both in the same breadth of one review, as they are approached differently from a clinical standpoint, are modeled differently in a research context, and have different pathohistological hallmarks. Similarly, as there are many parallels in ischemic stroke and acute myocardial infarction, it is likely that some of this review could apply to salvaging neurons. However, the inherent differences in sensitivity to ischemia between neurons and cardiac myocytes. Neurons begin to die after <10min of hypoxia while for cardiac myocytes in rat models, it takes 20 minutes of ischemia to observe irreversible cell injury. Furthermore, microenvironment differences (i.e. neurons are often immunoprivileged, bathed in CSF, surrounded by various neuroglia), cell signaling and metabolism are among other factors that may limit translatability.\textsuperscript{14,15}

Nrf2 is a novel therapeutic target in the context of acute MI, for which there are presently no clinical trials implementing targeting strategies in the ways this review will propose. In the context of protecting tissues from ischemia, reperfusion, and the ROS throughout, Nrf2’s merits appear to be many, especially when one considers the peri-infarct zone (PIZ). This portion of the myocardium is also considered ‘at-risk’, and can be differentiated from infarcted myocardium using a combination of early- and late- contrast-enhanced cardiac MRI.\textsuperscript{16} The extent of peri-infarct tissue scarring, again using MRI, has proven to be a powerful risk stratification tool to predict incidence of arrhythmia and overall mortality following acute MI.\textsuperscript{17} The degree of systemic inflammatory activity in the first five days post-MI is directly correlated with the size of the PIZ.\textsuperscript{18} The PIZ is subjected to latent insults such as microvascular obstruction (MVO) and inflammation -- wherein reactive oxygen species (ROS) play a significant role -- and experiences ongoing cell death following the surgical and/or medical reperfusion therapies of the day. The size and the fate of this at-risk tissue presently hang in the balance, being used more so as a predictive prognostic tool than a target itself. At the time of writing this review, only scant literature exists on explicitly targeting the PIZ therapeutically, but early efforts are encouraging.\textsuperscript{19} Efforts to minimize latent cell damage/death and maladaptive remodeling post-reperfusion should be at the forefront if we wish to further improve patient
outcomes and reduce to the impact acute MI has on patients and the healthcare system.

The span of Nrf2 research is immense, as it is the “master regulator of (oxidative) stress” and (oxidative) stress is a component, if not the root cause, of many disease states. Research regarding Nrf2 has revealed that myriad cellular pathways are involved, but this review focuses on the earliest and most defining events of acute MI ischemia and reperfusion, and proposes the rationale for Nrf2 targeted-therapies during these two critical stages.

**Cardiac Myocyte Function & Physiology Background**

Cardiac muscle cells (myocytes) are tasked with pumping blood throughout the pulmonary and systemic circulations for the entirety of a person’s life. They are typically predominantly mononucleated cells in humans, while they are predominantly polynucleated cells in rats and mice. Cardiac myocytes branch such that they connect end to end to neighboring myocytes. Histologically, these cells appear striated, as they are virtually packed with myofibrils -- concatenated sarcomeres -- the contractile units of striated muscle comprised of overlapping thin and thick filaments.

![Figure 1: Normal histology (using H&E stain) of cardiac muscle. Note the branching myocyte morphology, striations, (A) centrally-located nuclei, and (B) intercalated discs (gap junctions).](image)

Tucked between myofibrils and under the cell membrane are mitochondria, and this distribution makes ATP readily and rapidly available throughout the cell. ATP is essential for cross-bridge cycling between actin and myosin, which manifests as cellular contraction. The location and sheer number of mitochondria will be an important point to consider when we look into ischemia and reperfusion injury later. A very important organelle that is responsible for storage and shuttling of calcium, the sarcoplasmic reticulum, envelops the myofibrils. The cell membrane (also known as the “sarcolemma”) makes periodic deep invaginations called t-tubules into the cell. T-tubules are buttressed on either end by sarcoplasmic reticulum, and this subcellular structure is called a diad. This structural detail is important because it means that the all-or-none depolarizing action potential,
initially mediated by sodium flowing into the cytoplasm (also known as the “sarcoplasm”), spreads virtually instantaneously around and throughout the entire cell. The next step in eliciting cellular contraction immediately following sodium influx is the opening of voltage-gated calcium channels on the sarcolemma and the sarcoplasmic reticulum, causing a tremendous increase in cytoplasmic/sarcoplasmic [Ca^{2+}].

Figure 2: Increasingly closer looks at cardiac myocytes. (A) shows striated, branching cardiac myocytes. (B) provides a view of cardiac diads and the organization of myofibrils, sarcoplasmic reticulum, and mitochondria. (C) illustrates sarcomeres, the functional units of striated muscle comprised of (D) thick and thin filaments.

Along with ATP, calcium is an essential component to initiate actin-myosin cross bridge cycling and cell contraction; in the absence of calcium, the myosin binding sites on actin in thin filaments are covered by tropomyosin such that they are inaccessible to myosin. Tropomyosin is, in turn, bound to troponin, which has three subunits: troponin C (TnC), troponin T (TnT), and troponin I (TnI). TnC binds calcium, thus sensing the ‘switch’ of cytoplasmic calcium increase during an action potential. TnC calcium binding induces protein conformational changes that result in tropomyosin being pulled away, revealing the myosin binding-sites on actin, thus allowing cross bridge cycling to occur (if ATP is available).

Figure 3: A detailed view of the components of thick and thin filaments.
Cardiac myocytes live a perpetually demanding existence as they are long-lived, have little regenerative capacity, and must shuttle ions into or out of the cytoplasm and sarcoplasmic reticulum – against their concentration gradients – for virtually every heartbeat. This requires a tremendous amount of ATP, with the much more efficient means of obtaining ATP through oxidative phosphorylation being optimal. Over 95% of the ATP generated in the non-ischemic heart comes from oxidative phosphorylation, mostly fueled by fatty acid beta-oxidation.\(^\text{23}\) If the rate of cardiac ATP usage remained unchanged and ATP synthesis were suddenly ablated, ATP stores would be depleted within 15 seconds, due in part to the relatively low glycogen content in cardiac myocytes.\(^\text{24,25}\)

Ventricular myocytes are the cells responsible for pumping blood to the pulmonary and systemic circulation. A brief review of the phases of ventricular action potential illustrates the need for synchronicity as well as the energetic demands in this process (see figure below). These changes are mediated by the stepwise and synchronous opening and closing of ion channels, which is initially triggered by pacemaker cells and propagated throughout the cardiac muscle via gap junctions. Due to gap junctions, cardiac muscle is often described as a syncytium.\(^\text{26}\)
**Figure 5:** The ventricular cardiac myocyte action potential stages are as follows. Phase 4 is resting membrane potential (~90mV) at which time cells are depolarized and in diastole. This is attributed to the potassium equilibrium potential (which is in turn driven by the sodium-potassium ATPase). At this time, potassium leak \( I_{K1} \) channels are open and both fast-sodium channels and slow (L-type) calcium channels are closed. Pacemaker cell depolarization (not pictured) is conveyed through gap junctions, initiating all-or-none depolarization (Phase 0) by inducing fast sodium channels \( I_{Na} \) to open and K-leak channels to close. At ~40mV, L-type calcium channels open \( I_{Ca(L)} \). Phase 1 signifies closing of the fast-sodium channels and opening of a distinct outward potassium channel \( I_{Kto} \), causing a slight repolarization that appears as a “notch” in the action potential. Phase 2 is driven by sustained opening of L-type calcium channels, which creates a plateau in the action potential. Excitation-contraction coupling occurs at this time, as calcium-induced calcium release from the sarcoplasmic reticulum facilitates actin-myosin crossbridge cycling. L-type calcium channels spontaneously close, K-leak channels reopen, and the cell repolarizes in phase 3.  

The figure below, summarizing the pertinent ion channels involved in cardiac depolarization, excitation-contraction coupling, and repolarization rounds out our baseline review of cardiac physiology. With this background, we can begin to delve into the aberrations that occur in these processes as a result of ischemia and/or reperfusion.

**Figure 6:** Depiction of pertinent ion channels in cardiac myocytes. Depolarization initiated by pacemaker cells ripple through cardiac myocytes via gap junctions. This is initially conveyed through the opening of (1) Sodium voltage-gated channels followed by (2a) L-type calcium channels (open at ~40mV). Increased Sarcoplasmic calcium concentration causes calcium-induced calcium release from the sarcoplasmic reticulum via (2b) Ryanodine receptor. At this...
time, cell contraction is actively occurring. Repolarization occurs as a result of (3) Potassium leak channels opening and ion homeostasis being re-established via the (4a) Sodium-Calcium exchanger, ATP-dependent Calcium sequestration through (4b) SERCA (Sarco/Endoplasmic Reticulum Calcium ATPase), and (4c) sodium-potassium ATPase. In the event of cellular acidosis, which occurs as a result of ischemia, the (5) sodium-hydrogen exchange channel becomes critical as at the expense of extruding protons, it disrupts the aforementioned synchronized balance of other key ions (sodium & calcium) and therefore, impairs cellular function.\(^\text{27}\)

Ischemia-induced cardiac injury is a dynamic process whereby initial, reversible changes and damage segue into irreversible cell death and eventual replacement with a fibrous scar. ROS play an integral role throughout pathological ischemia and reperfusion. As such, a case will be made that cell viability (and by extension patient outcomes) would improve if Nrf2 transcriptional activity could be therapeutically upregulated in some manner. Furthermore, the earlier Nrf2-inducible gene products are increased, the better; there will be a clear distinction between the benefit from pre-emptive Nrf2-based therapies and cells that are “catching up” after ischemia-reperfusion.

**Role of the Electron Transport Chain**

Review of oxidative phosphorylation – specifically the electron transport chain (ETC) – serves a dual purpose. It is both the driving force behind the bulk of ATP generation in cardiac myocytes and introduces one main mechanism of ROS created under both physiological and pathological conditions. As a result of glycolysis and the citric acid cycle, NAD and FAD are reduced to NADH and FADH\(_2\); converting one glucose molecule to CO\(_2\) via glycolysis and the citric acid cycle yields 10 NADH and 2 FADH\(_2\) molecules. NADH and FADH\(_2\) are then oxidized by complex I and II of the ETC, respectively. The ETC is a series of proteins and protein complexes with increasing reduction potential (i.e. affinity for electrons; the higher the potential, the higher the electron affinity) in the inner mitochondrial membrane, which separates the mitochondrial matrix from the intermembrane space. As electrons pass through the ETC complexes in incrementally exergonic (-\(\Delta G\)) steps, energy is used to drive protons against an electrochemical gradient from the mitochondrial matrix to the intermembrane space in complexes I, III, and IV. The result is a proton-motive force, which is then used to power the ATP synthase (complex V).\(^\text{28}\)
Figure 7: A schematic view of glycolysis, the citric acid cycle, fatty acid oxidation, the electron transport chain, and the ATP synthase as they pertain to oxidative phosphorylation in the mitochondria.\(^{29}\)

Figure 8: There is gradual increase in reduction potential and decrease in free energy (\(\Delta G\)) along the ETC. Electrons from NADH (abstracted from NADH dehydrogenase; complex I) and FADH\(_2\) (abstracted from succinate dehydrogenase; complex II) are transferred to ubiquinone (coenzyme Q; CoQ) to form reduced ubiquinol (CoQH\(_2\)). In complex III (CoQH\(_2\)-cytochrome c reductase), electrons are transferred from CoQH\(_2\) to cytochrome c, in a slightly convoluted manner called the Q cycle. Reduced cytochrome c then moves to complex IV (cytochrome c oxidase), where four equivalents of electrons from of cytochrome c are transferred to O\(_2\) to form H\(_2\)O.\(^{29}\)

Molecular oxygen (O\(_2\)) has a greater reduction potential (higher electron affinity) than any of the other ETC components, and is usually the last stop of the electron transport chain, whereby O\(_2\) is reduced to water (H\(_2\)O) at complex IV. Oxygen in the "ground (triplet) state" has two unpaired electrons in the highest energy orbitals – this means it actually exists as a biradical. Following the Pauli exclusion principle and Hund’s rule of electron orbital filling, these unoccupied orbitals must be filled in one electron at a time; O\(_2\) reduction to H\(_2\)O happens in four one-electron transfer steps\(^{30}\).
First: $O_2 + 1e^- \rightarrow O_2^-$ (superoxide radical)
Second: $O_2^- + 1e^- + 2H^+ \rightarrow H_2O_2$ (hydrogen peroxide)
Third: $H_2O_2 + 1e^- + 1H^+ \rightarrow H_2O + \cdot OH$ (water and hydroxyl radical)
Fourth (final): $\cdot OH + 1e^- + 1H^+ \rightarrow H_2O$

**Figure 9:** Stepwise Electron Reductions from $O_2$ to $H_2O$, taken from "Free radicals and disease in man".

As electrons are added, the intermediates are increasingly unstable up until finally arriving at water. Harkening to the principle of bond order best depicts this:

$$\text{Bond order} = \frac{\text{# bonding electrons} - \text{# anti-bonding electrons}}{2}$$

The larger the bond order, the closer and more stable the bond, and vice versa. Comparing the molecular orbital diagrams below (antibonding orbitals indicated by asterisks and antibonding electrons in red) of increasingly reduced oxygen intermediate redox states gives the following bond orders: ground state oxygen = 2 (most stable); superoxide radical anion = 1.5; peroxide ion = 1 (least stable).

**Figure 10:** Molecular orbital diagrams (simplified; not showing 1s or 2s orbitals) for various oxygen redox states. As electrons are added, anti-bonding orbitals fill and bond order decreases. Molecular oxygen ($O_2$) at ground state exists as a diradical.

Similar to the step-wise nature of $O_2$ reduction, components in the ETC are also reduced or oxidized one electron at a time. For example, ubisemiquinone (QH$^-$) is a radical intermediate between ubiquinone (Q) and ubiquinol (QH$_2$).
The ETC is not a perfect machine, and premature 'leaking' of electrons to O₂ as they pass through the ETC intermediates prior to reaching complex IV does occur, forming superoxide (O₂⁻), the "parent" radical for ROS. This is most thoroughly documented in complexes I and III. Using isolated mitochondria under (resting) physiologic conditions, it has been shown that only ~2% of the electrons passing through the ETC contribute to superoxide formation in vitro. This is still likely an overestimate as mitochondrial oxygen concentration is lower than atmospheric concentration in vivo and therefore likely scarcer than the in vitro experimental model).

**Reactive Oxygen & Nitrogen Species**

This review’s aim with regard to ROS/RNS is to describe the basics of how they affect the macromolecules in cells such as proteins, nucleic acids, and lipids. Note that the word ‘affect’ was used and not ‘damage’, because at low levels, ROS/RNS serve as physiological second messengers with macromolecule modifying potential, and this is not necessarily always harmful. "Physiologic" concentrations of ROS are considered essential components of many cell signaling pathways pertaining to metabolism, growth, and survival.

Reactive oxygen and nitrogen species (ROS, RNS) can certainly impart significant cellular stress in high concentrations. One instance of physiologic...
levels of high ROS production is in neutrophils, the “first responders” of inflammation. These cells create extraordinary levels of superoxide using the enzyme NADPH oxidase, which causes damage/death to pathogens (and also to host cells in proximity). The environment within a cardiomyocyte facilitates pathological levels of ROS from within during ischemia and reperfusion, which can be followed by cell death. Certain forms of cell death, in turn, can elicit a “sterile” immune response (including tissue infiltration by neutrophils). This perpetuates the problem at hand, as neutrophils can leak harmful ROS and impact healthy cells living on the edge of the infarct.35

There are many articles and books solely on ROS/RNS production, as there are a multitude of potential sources and a high degree of complexity with regard to these molecules and their physiological and pathological roles. All species depicted below are reactive, but not equally reactive. For instance, hydroxyl radical (\(\cdot OH\)) has a reduction potential (electron affinity) ~2.5x greater than superoxide radical (\(\cdot O_2^-\)). Some reviews have posited that being the most reactive ROS does not necessarily make it the most harmful. In most cells, radicals are physiologically (and pathologically, as we will see) produced predominantly in the mitochondria. Abstractly speaking, if an organism lives through an oxidative stress episode, DNA damage would be considered the most harmful; proteins and lipids can be (and are) turned over with little consequence. The extreme reactivity of hydroxyl radical means it will almost certainly exert its effect on a macromolecule before arriving at the nucleus to damage DNA. Another introductory point is that whether or not a ROS/RNS entity is a radical does not predict reactivity; non-radical ROS/RNS molecules readily react in cells such that their products form radicals.36

![Figure 13: A scheme of reactive oxygen and nitrogen species, with relative reactivity (in the form of reduction potentials) shown in red.]

As we will discuss in the next section, the ETC can generate a profound amount of ROS, especially if it becomes uncoupled and electron flux is shunted prematurely to oxygen instead of the typical intermediate. Take the Q cycle, which occurs in complex III, for example:
Figure 14: A simplified Q cycle scheme, as it pertains to ROS formation.

Single electron transfers cycle between ubiquinone (Q), semiubiquinone (Q\textsuperscript{-}) and ubiquinol (QH\textsubscript{2}), with the potential for single electron transfers to be directed to O\textsubscript{2} in the event that ubiquinone stores are all reduced and unable to accept additional electrons.\textsuperscript{37}

Reactive oxygen species can interact with DNA in a number of ways, including induction of intra- and inter-strand and protein crosslinks, causing single and double-strand breaks, and modifying bases.\textsuperscript{38} RNA, which is in closer proximity to the mitochondria than DNA and does not have parallel repair mechanisms to DNA, is even more susceptible to ROS modification.\textsuperscript{36} Mitochondrial DNA (mtDNA) is closer to the point of generation of ROS and is therefore more susceptible to damage than nucleic DNA. A 4-week post-MI mouse model, wherein the LAD artery was permanently ligated and the left ventricle subsequently dilated, exhibited decreased contractility, and histologically showed hypertrophy and interstitial fibrosis. These changes are consistent with heart failure secondary to MI. Among the molecular changes demonstrated were a decrease in mtDNA, mtRNA, as well as decreased expression and activity of mt-encoded enzymes complexes I, III, and IV in MI mice. There was also a greater degree of ROS production and lipid peroxidation in MI mice compared to controls, indicating a vicious cycle of mitochondrial dysfunction and ROS generation, even in the tissue that survives 4 weeks after LAD occlusion.\textsuperscript{39} DNA damage following acute MI is also reflected in humans treated with either thrombolytic therapy or PCI.\textsuperscript{40} It is worth noting that the marker used in the cited study, 8-hydroxydeoxyguanosine, does not differentiate between mitochondrial and nucleic DNA damage and is not cardiac-specific. It is the opinion of the author that despite those limitations, these studies – among many others that will not be reviewed in detail – have done a sufficient job in showing that cardiac nucleic acids are compromised by ROS during and after acute MI.

ROS can change protein structure by inducing cross-linking, altering the overall charge of the protein, and even causing fragmentation of the peptide chain. These changes in the primary protein structure can obviously alter secondary,
tertiary, and quaternary structures. Some R-group alterations, such as protein carbonylation, are a budding topic of study and some debate in terms of impact. There are physiological and pathological elevations of protein carbonylation, but how to discern between the two is not very clear. Furthermore, some publications report no change in protein function as a result of carbonylation while others propose it to be a novel mechanism of dysfunction. The truth of the matter, whatever it may be, remains unclear at this time.

On the other hand, there is a strong consensus that other post-translational modifications of proteins by ROS can be sensed and serve as switches, much like phosphorylating a protein. For instance, we will see later how critical cysteine residues can ‘sense’ and thereby regulate the stress response of Nrf2. Some examples of sulfur modifications are depicted below – note that many of the modifications are reversible, but some induced by ROS are not.

![Figure 15: Potential modifications of cysteine. The red area indicates irreversible modification.](image)

Lastly, lipids can be greatly altered by ROS. Any reaction between a radical species and a non-radical species will create a radical species reaction product. Therefore radicals are prone to setting off chain reactions. Electrons can be abstracted from polyunsaturated fatty acid tails, and generate hydrocarbon free radicals, which react with O₂ to form peroxyl radicals, which can abstract an electron from a nearby polyunsaturated fatty acid tail, generating a lipid hydroperoxide and yet another lipid radical. Phospholipids create discrete barriers by forming membranous structures, and their amphipathic nature is essential to establish barriers. Polar lipid hydroperoxides are not as stable in what was once a very non-polar hydrocarbon environment in the phospholipid bilayer. Extensive lipid peroxidation results in loss of membrane integrity (mitochondrial and cellular), swelling, and cell death.
Key Events in Early Ischemia

In 1999, researchers at the University of Chicago elucidated the culprits of ROS generation during a one-hour bout of ischemia by using ETC inhibitors for specific proteins, and complexes I and III were identified as the major sources. In their experimental model using chick cardiomyocytes, the researchers were able to obtain intracellular oxygen concentration levels lower than any reported whole-animal model, yet still observed ROS generation. It may seem initially counter-intuitive that a modest increase in ROS is generated during what is, by definition, a time of oxygen scarcity. Interestingly, notable known enzymes with ROS-generating potential such as xanthine oxidase, nitric oxide synthase, and NADPH oxidase were essentially non-factors in the first hour of ischemia.
Ischemic levels of oxygen will preclude cytochrome oxidase (complex IV) from passing electrons to the ultimate intended recipient of the ETC (O2), and electron flux from cytochrome c is halted.\textsuperscript{49} The ETC gets “backed up” and reduced intermediates such as NADH and ubisemiquinone also accumulate.

Complex I can exist in active (A) or de-active (D) forms and which form predominates is depending on the oxygenation level. The population of Complex I predominantly in the A-state under normoxic conditions (87%) but becomes predominantly deactivated (only 40% active) during anoxia. The activity reverts back upon reoxygenation, but the authors were able to keep complex I predominantly inactive, even in the presence of oxygen, by keeping the ubiquinone pool reduced. This raises a critical question: if the ubiquinone pool is reduced and unable to accept any more electrons in hypoxia, how is there still 40% activity of the enzyme? Where are the electrons going? Authors concluded that O2 is almost certainly the (premature) recipient after using cyanide, a Complex III inhibitor, and still observing 32% activity of complex I.\textsuperscript{50}

As of 2005 it was apparent that Complex III is a major source of ROS production during ischemia and reperfusion. Moreover, it dwarfs complex I’s ROS contribution. Fluorescence resonance energy transfer (FRET) was implemented to sense cytosolic ROS in live cells. The sensing protein consisted of cyan and yellow fluorescent proteins (CFP/YFP) linked to the redox-regulated heat-shock protein HSP-33 such that oxidation of a thiol in HSP-33 caused CFP/YFP dissociation and subsequent loss of fluorescence. shRNA against the complex III-specific Rieske iron-sulfur protein (RISP), which normally transfers an electron from ubiquinol to cytochrome c1, as well as other complex III inhibitors (stigmatellin and myxothiazol) all attenuated hypoxic ROS based on FRET findings, though they did not completely abolish ROS generation (there is still ROS coming from complex I at this time). The authors determined that complex III must be the predominant source of cytosolic ROS because they did not alter complex I whatsoever and only when complex III was active was there sufficient H2O2 (which can be made rapidly from superoxide via SOD) to activate HIF-1a. Rotenone, a complex I inhibitor, is able to block HIF-1a signaling in normoxia, but Rotenone treatment during hypoxia had no effect, indicating that complex III creates the lion’s share of ROS during ischemia. This is likely due to complex I having the previously mentioned active-deactive conformational distinction and becoming less active during ischemic conditions.\textsuperscript{51}
Figure 18: FRET-Hsp-33 ROS sensing shows hypoxia-induced ROS generation is mostly a product of complex III activity, as indicated by attenuation with shRNA inhibition of Rieske iron-sulfur protein (RISP), a complex III protein. Note that even with shRNA to RISP, hypoxia still increases ROS generation (red line). As a bit of foreshadowing, also note that in the WT (Black line), reperfusion indicates ever-increasing ROS rather than a return to baseline.\textsuperscript{51}

Taken together, these studies and others have confirmed that ischemic uncoupling of the ETC results in complexes I and III performing single-electron transfers to O\textsubscript{2} (either directly or indirectly via the intermediates ubiquinone and cytochrome c), forming moderate amounts of superoxide.\textsuperscript{37,52}

Cardiolipin is an anionic phospholipid localized to the inner mitochondrial membrane, and is essential for proper functioning of the ETC. It serves as a scaffold for complexes III and IV, as well as 'super complexes' consisting of trimers or tetramers of complexes III and IV.\textsuperscript{53} Using submitochondrial particles, it has been observed that ROS-driven lipid peroxidation causes a loss of cardiolipin. Decreased cardiolipin content following ROS exposure is directly related to loss of complex IV activity, proven by the attenuation from pretreatment with excess exogenous SOD/Catalase, recovery of function following addition of cardiolipin, and no effect following addition of peroxidized cardiolipin.\textsuperscript{54} Cardiolipin also binds cytochrome c, keeping it associated with the inner mitochondrial membrane. Localizing cytochrome c is necessary for ETC function, and cytoplasmic cytochrome c can associate with Apaf-1 and form the apoptosome in intrinsic, caspase-dependent apoptosis. Lipid peroxidation of cardiolipin causes dissociation of cytochrome c into the cytoplasm and apoptosis ensues, but this can be rescued by addition of exogenous cardiolipin.\textsuperscript{55} Because lipid peroxidation is a chain reaction until it is quenched, even the modest increase in ROS during ischemia can pose a threat. If antioxidant defenses are overwhelmed, cardiolipin can rapidly peroxidize, compromising the inner mitochondrial membrane (and thus, membrane potential) and rendering complexes III, IV, and cytochrome c unable to carry out their normal functions. If and when these cells are reperfused, it is not difficult to imagine chaos ensuing with regards to ROS formation and bioenergetics.
Rapid ATP depletion after ischemic onset prohibits proper contractility and the 3Na⁺/2K⁺ ATPase no longer maintains the normal electrochemical gradients for these two key ions. The Ca²⁺ gradient is then lost via the Na⁺/Ca²⁺ exchanger, which requires no ATP and remains functional during ischemia. At this point, every critical ion gradient covered in the review of cardiomyocyte physiology goes askew and the cell depolarizes. Despite being depolarized, cells have no ATP to use for crossbridge cycling. Reversible rigor-like contractures have been observed under low-ATP conditions in isolated ischemic myocytes. Once ischemic, the myocardium rapidly shifts (within one minute) to low-efficiency anaerobic glycolysis to generate ATP, and CO₂, H⁺, and lactate build up within cells as byproducts. Upon reperfusion, this acidosis will prove to be of utmost importance. A lactate-H⁺ cotransporter exists (monocarboxylate transporter 4; MCT4) to allow efflux of these two entities that accumulate during ischemia. After 30 minutes of ischemia, MCT4 mRNA and protein increase and lactate concentrations in cardiac effluent increase from near 0 mmol/L during normoxia to 1.5 mmol/L after 30 minutes ischemia, 0.4 mmol/L at 15 minutes of reperfusion, and return baseline after 60 minutes of reperfusion. However, MCT4 has a low affinity – one study using skeletal muscle reports a Kᵡ of 20milliM – indicating it is only active at extremes. For reference, isolated rat hearts pre-treated with 10mM lactate perfusate then subjected to ischemia causes a 60% stall in glycolytic flux and complete myocardial stunning, contrasted with 10mM pyruvate and 10mM glucose perfused hearts.

To recap, key points of ischemia reviewed here are a switch from oxidative phosphorylation to glycolysis, intracellular acidosis, an increase in the NADH/NAD⁺ ratio, ATP depletion, loss of ionic homeostasis (including mitochondrial depolarization), loss of contractility, electron transport chain uncoupling, formation of a modest amount of ROS (predominantly via complexes I and III), and these ROS can damage lipids, proteins, and nucleic acids.

Cells subjected to prolonged ischemia without reperfusion will undergo coagulative necrosis. There are no pathohistological changes in the first 4 hours of ischemia, which gives a visual indication that the fate of the tissue may not yet sealed. From 4 to 24 hours, however, coagulative necrosis hallmarks become appreciable, which are depicted and described below.
Figure 20: Coagulative necrosis of cardiac muscle, 4-12 hours after infarction. Visual hallmarks include loss of myocyte nuclei and striations, and often signs of hemorrhage (more interstitial RBCs present than in normal tissue).\textsuperscript{20,62}

**Ischemia-Reperfusion Injury**

Upon reperfusion, ATP can be synthesized and used to attempt to normalize gradients. Although cells are attempting their return to normality, the changes that took place during ischemia – namely acidosis, calcium overloading, and ETC uncoupling – will only take full effect upon reperfusion. The mitochondrial permeability transition pore (MPTP) is a non-selective pore permissible to ions and molecules up to 1 kDa that opens in response to very high (pathologic) $[\text{Ca}^{2+}]_{\text{mtm}}$, such as in the case of ischemia.\textsuperscript{63} However, the acidotic environment during ischemia prevents opening of the MPTP.\textsuperscript{64,65}

The mitochondrial outer membrane is freely permeable to calcium. The inner membrane uses a channel called the mitochondrial calcium uniporter (MCU) for flux into the mitochondrial matrix, but only when calcium concentrations are very high.\textsuperscript{66,67} Transient increased matrix mitochondrial calcium ($[\text{Ca}^{2+}]_{\text{mtm}}$) serves as a physiologic signal in normal conditions, increasing TCA cycle flux and activity of ETC complexes, causing an rise in ATP production.\textsuperscript{67,68,69,70} This is an entirely rational response for cardiac myocytes under normal conditions, as high cytoplasmic calcium would indicate the cell is depolarized for systole and ATP is needed for the desired contraction. Isolated mitochondria in physiologic conditions do not depolarize following transient increases in cytoplasmic calcium concentration.\textsuperscript{71} During ischemia, however, when ATP-dependent ionic housekeeping is compromised throughout the cell and it is depolarized, mitochondria are subjected to sustained high $[\text{Ca}^{2+}]_{\text{mtm}}$, which promotes formation and opening of the mitochondrial permeability transition pore (MPTP).\textsuperscript{72}

Another consequence of sustained high intracellular calcium during the early phase of MI is the activation of the calcium-dependent protease, calpain, which can result in irreversible damage to cells.\textsuperscript{73} Calpain inhibitors administered to rabbit hearts during reperfusion conferred increased recovery of cardiac function and integrity of mitochondrial membranes.\textsuperscript{74} Calpain activation during reperfusion injury is a result of the
reintroduction of ATP.\textsuperscript{75} One way calpain can damage cells is by cleaving xanthine dehydrogenase (an enzyme involved in purine metabolism), irreversibly converting the enzyme to xanthine oxidase, which can create $\text{O}_2^- \text{ and } \text{H}_2\text{O}_2$ directly. Xanthine oxidase is indeed elevated in MI patients, along with serum levels of malondialdehyde, a product of lipid peroxidation ($p<0.0005$ for both findings).\textsuperscript{76}

Intracellular acidosis established during the ischemic period stimulates extrusion of $\text{H}^+$ via the $\text{Na}^+/\text{H}^+$ exchanger and the $\text{Na}/\text{HCO}_3^-$ symporter. These channels collectively extrude $\text{H}^+$ while increasing intracellular bicarbonate and sodium – recall that sodium depolarizes cells and activates voltage-gated calcium channels. As oxidative phosphorylation resumes and ATP levels rise, the high $[\text{Ca}^{2+}]_{\text{cytosol}}$ causes contractile dysfunction in the form of hypercontractility. Pathology findings will reflect dense contraction bands. Reperfusion causes the pH to rapidly normalize, which in turn allows for MPTP opening, and concentration gradients such as those that create the mitochondrial membrane potential ($\Delta\psi_m$) are lost and the mitochondrion depolarizes. Without the proton motive force to be used by ATP synthase, oxidative phosphorylation cannot occur. The high protein concentration within the mitochondria (colloidal pressure) can also cause influx of water, mitochondrial swelling, and even rupture. In the event of hypothetical global mitochondrial rupture, further metabolic and ionic dysregulation along with massive cytoplasmic cytochrome c can result in either necrotic or apoptotic cell death. Note that apoptosis requires ATP. If only partial mitochondrial rupture occurs, the increased cytochrome c can affect the intrinsic, caspase-dependent mode of apoptosis, which will be fueled by ATP from the remaining intact mitochondria. Because MPTP opening is prevented at low pH, gradual normalization (as opposed to sudden) of perfusate pH has been a proposed tactic to avoid IRI. Another potential benefit of maintaining acidic pH is that proteases such as calpain lose function in acidic conditions, and a rapid return to normal pH will release this inhibition.\textsuperscript{77,78} Because ETC complexes I and III tend to be uncoupled following pathologic ischemia, they essentially become \textit{de facto} ROS-generating entities. This makes gradual re-introduction of oxygen to the tissue is another attractive strategy, as cells likely have a better chance of managing and living through the reperfusion-induced ROS if it occurs gradually (as opposed to the burst that presently occurs). Both of these principles have been proven in principle via experiments and are parts of what is collectively known as post-ischemic conditioning.\textsuperscript{79,80,81}

Cells that have persevered up to this point are by no means out of the woods, so to speak, and neither are their neighbors. An acute inflammatory response, which entails neutrophil and macrophage infiltration to the tissue, is mounted to respond to death signals from necrotic cells.\textsuperscript{82} These inflammatory cells (especially neutrophils) can be sources of ROS and collateral damage to bystanders, and oxidative stress is now both an intracellular and extracellular problem.
Microparticles (MPs), small membrane vesicles released from endothelial cells after ROS exposure, can perpetuate events of ischemia, reperfusion, and inflammation by obstructing flow in downstream capillaries. MPs can travel through capillaries, which are typically ~5 micrometers in diameter and can aggregate with platelets, with the potential to become nucleation sites for larger complexes. In the last five years, microparticles have been shown to be associated with microvascular obstruction (MVO) and no reflow phenomenon. No reflow phenomenon refers to tissue remaining ischemic despite luminal opening of the previously occluded artery. The glycocalyx which sits upon the lumen-facing side of vascular endothelial cells is also implicated in ischemia reperfusion injury in that it deteriorates following ischemia-reperfusion, coinciding with impaired endothelium-mediated vasodilation – effects driven by ROS to a degree, as they are attenuated with inhibition of xanthine oxidase. Precisely how ROS contribute to initiating this cascade is still being investigated, but it makes ROS-directed therapeutic strategies all the more attractive.

![Figure 21: Some of the factors during ischemia and reperfusion.](image)

![Figure 22: Contraction band necrosis, observed following reperfusion. This example is likely before ~12 hours, as there is not yet marked neutrophil or macrophage infiltrate. Nuclei are no longer visible in the myocytes and there are (A) dense contraction bands where sarcomeres are hypercontracted.](image)
Figure 23: After 72hrs, the acute inflammatory response (neutrophil and macrophage infiltrate) is abundantly clear. The combined effects of ROS generated from dead cardiomyocytes and inflammatory cells can potentially perpetuate the ischemia-reperfusion injury cycle in surrounding tissues.  

Figure 24: Between 1-3 weeks, there is a robust fibrovascular response that appears as “granulation tissue”. Note the sprouting capillaries and the proliferating fibroblasts, laying down extracellular matrix: a non-contractile scar. This loss of conducting, contractile tissue is a major contributing factor in the susceptibility to developing problems such as heart failure and arrhythmias post-MI. Limiting the extent to which cardiac muscle tissue lost and replaced with non-functioning scar tissue is an ongoing global clinical and research effort, and the motivation behind this review.

Nrf2 History & Structure

The discovery of Nrf2, the transcription factor often called the “master regulator of oxidative stress,” initially came about due to interest in the processes of drug metabolism, particularly in phase II of drug metabolism or ‘conjugation’ of xenobiotics. In 1990, it was discovered that a discrete regulatory element on DNA upstream (5’ adjacent promoter) of phase II drug metabolism enzymes responded to the bifunctional (phase I and phase II enzyme) inducer B-naphthoflavone as well as to the phenolic antioxidants t-butyldihydroquinone (t-BHQ) and butylated hydroxyanisole (BHA) in a monofunctional manner. This cis regulatory element was aptly named the antioxidant response element (ARE). This element’s consensus sequence was further elucidated in 1997 and is upstream of not only phase II drug metabolism enzymes, but also more
broadly a battery of cytoprotective genes coding for antioxidant enzymes which are induced by reactive oxygen and nitrogen species (ROS, RNS) as well as chemical compounds with the capacity to become electrophilic intermediates via redox cycling or metabolic transformation.\textsuperscript{92,93}

Moi et al were the first to isolate Nrf2 in 1994 as a novel bZip transcription factor that bound to the beta-globin gene locus\textsuperscript{94}; at the time, nobody had considered Nrf2 as it pertains to ARE or oxidative stress. Itoh et al determined in 1997 that Nrf2 (as a heterodimer with small Maf transcription factor) is the transcription factor responsible for binding to the ARE and inducing detoxifying phase II enzymes such as glutathione S-transferase (GST) and NAD(P)H quinone oxidoreductase (NQO1).\textsuperscript{95} Their group noticed the similarity between consensus sequences of ARE and the binding site for erythroid transcription factor NF-E2. NF-E2 (p45) must form a heterodimer with a small Maf (sMaf) protein to induce transcription. Maf proteins can be categorized as ‘bZip’ proteins in that they contain conserved basic domains and leucine zipper domains -- the former binds DNA and the latter mediates homo- or heterodimerization with other bZIP transcription factors.\textsuperscript{96} The bZip domain was first discovered in the cap'n'collar (cnc) transcription factor in \textit{Drosophila}, so named due to its necessity during differentiation of the \textit{Drosophila} head/jaw segment during development. Proteins subsequently discovered with similar motifs fell into the “CNC family”.\textsuperscript{97} Itoh et al established four candidate CNC proteins -- Nrf1, Nrf2, ECH, and Bach -- to explore as potential ARE transcription factors. The list narrowed down to Nrf2 after reviewing the work of others or doing primary research to rule out the other candidate transcription factors based on their expression profiles, i.e. the other candidate transcription factors when constituitively expressed did not upregulate gene expression of the aforementioned phase II enzymes of immediate interest. They succeeded in determining Nrf2 was responsible for inducing genes with 5'-adjacent AREs by mutating murine Nrf2 and showing loss of phase II gene induction from known chemopreventive (ARE-associated gene inducing) compounds.\textsuperscript{95} Ramos-Gomez et al published additional findings of high clinical relevance in 2001, showing Nrf2-deficient (knockout) mice expressed low levels of phase II detoxifying enzymes and were sensitized to carcinogenesis.\textsuperscript{98} The floodgates opened shortly thereafter with respect to publications concerning Nrf2.
Indeed, there are many publications showing the importance of Nrf2 in the context of myocardial health and disease, including myocardial infarction (to be described in subsequent sections). And yet, there are no clinical trials to date explicitly exploring exogenous Nrf2 activation during or after reperfusion in acute MI. This review aims to encourage and inspire interest on that front in the immediate future.

An overview of the structure of Nrf2 reveals a 605 amino acid protein comprised of six highly-conserved Nrf-ECH homology (Neh) domains which are essential for the function and regulation of Nrf2. From amino (N) terminus to carboxy (C) terminus, the Neh domains are as follows:

- **Neh2**: contains the DLG and ETGE motifs, which are necessary for Kelch-like ECH-associated protein 1 (Keap1) binding. Keap1 is part of a Cul3 E3 ubiquitin ligase and acts to negatively regulate Nrf2 transcriptional activity through degradation via the 26s proteasome.
- **Neh4 and Neh5**: bind to the coactivators c-AMP-response element binding protein (CBP) and Brahma-related gene 1 (BRG1) to activate transcription.
- **Neh6**: contains redox-insensitive binding sites for Skp1-Cul1-Rbx1/Roc1 ubiquitin ligase complex; it allows for turnover of Nrf2 even in oxidative stress conditions. This domain contains a phosphorylation site which, when phosphorylated by glycogen synthase kinase-3 (GSK-3), has a greater propensity to be bound and tagged for degradation by the aforementioned ubiquitin ligase complex.

![PubMed Search Results for Publications Containing "Nrf2"](chart.png)

**Figure 25**: Nrf2 publications (total) as a function of time.

**Figure 26**: Nrf2 domains
• Neh1: contains CNC-bZip region, which is responsible for DNA binding and protein dimerization
• Neh3: carboxy-terminal region that is necessary for transcriptional activation and may act as a transactivation domain.

Nrf2 contains a number of nuclear localization sequences (NLS) and nuclear export sequences (NES) (see cartoon above), which can be bound by importins and exportins respectively.

A relatively unique feature within the Nrf2 mRNA structure is an internal ribosomal entry site (IRES) in the 5′ untranslated region (UTR), which contains an 18S ribosomal binding site. Upon oxidative stress exposure, de novo Nrf2 expression can be initiated via this mechanism. This speaks to the importance of Nrf2 when cells undergo stressful stimuli; some stressors, including oxidative stress (and viral infection, heat shock, nutrient deprivation, accumulation of misfolded proteins, changes in intracellular calcium, and induction of apoptosis), can cause a marked decrease in global protein expression by precluding 5′-cap-mediated translation. This can be attributed to phosphorylation the alpha subunit of eukaryotic initiation factor 2 (eIF2). Cells undergo rapid (within 10 minutes) translational reprogramming and shift to IRES-mediated translation. Only a small subset of genes will encode an mRNA with an IRES, indicating the importance of said genes when cells enter an ‘emergency response’ mode. Under stressed situations that favor IRES-mediated translation, PI3K/AKT are essential for Nrf2 protein translation and subsequent ARE-associated gene induction in a variety of cell types. Modeling oxidative stress in yeast shows that cells can sense a spectrum of the oxidative stress burden, with moderate levels inducing the IRES-mediated transcriptional reprogramming changes described, but very high levels causing an association of IRES-containing mRNAs to be associated with polysomes yet not actually being actively translated. The notion of a spectrum of stress and an upper-limit of stress that a cell will persevere through is extremely important when viewed through the lens of acute MI, subsequent IRI, and attempting to salvage even more tissue than the gold-standard therapies of the day allow.

Genes Upregulated by Nrf2

Nrf2 induces a battery of genes with ARE in their promoter which encode cytoprotective enzymes. In this review we will narrow the focus to a select group of antioxidant and phase II enzymes whose ability to dampen ROS-mediated damage is immediately apparent. A paper from 2005, yet again by the Yamamoto group and colleagues, showed that the enzymes superoxide dismutase (SOD), catalase, glutathione reductase (GR), GSH S-transferase (GST), and NAD(P)H:quinone oxidoreductase (NQO1) exhibit decreased expression and/or activity and/or inducibility with the Nrf2 inducer 3H-1, 2-dithiole-3-thione (D3T) in Nrf2-/- mice compared to WT. Xanthine oxidase (XO) mediated cytotoxicity was greatly reduced in WT cells pre-treated with D3T.
This study and others like it, such as those characterizing Nrf2 gene-induction library with microarrays or ChIP-Seq buttress the theme that Nrf2 is protective and beneficial in virtually every tissue studied.\textsuperscript{114,115}

In keeping with our metabolic angle throughout this review, Nrf2 also upregulates enzymes that produce NADH, which is necessary to replenish glutathione and thioredoxin after they have quenched (i.e. reduced) ROS.\textsuperscript{116}

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide Dismutase (SOD)</td>
<td>Superoxide (O\textsuperscript{−}) (\rightarrow) O\textsubscript{2} or H\textsubscript{2}O\textsubscript{2}</td>
</tr>
<tr>
<td>Catalase</td>
<td>2 H\textsubscript{2}O\textsubscript{2} (\rightarrow) 2 H\textsubscript{2}O + O\textsubscript{2}</td>
</tr>
<tr>
<td>Glutathione Reductase (GR)</td>
<td>Reduce glutathione (GS-SG (\rightarrow) 2 GSH)</td>
</tr>
<tr>
<td>Glutathione S-Transferase (GST)</td>
<td>X + GSH (\rightarrow) GS-X</td>
</tr>
<tr>
<td>Glutathione Peroxidase (GPx)</td>
<td>2 GSH + H\textsubscript{2}O\textsubscript{2} (\rightarrow) GS-SG + 2H\textsubscript{2}O</td>
</tr>
<tr>
<td>Glutamate Cysteine Ligase Catalytic (GCLC)</td>
<td>Glutathione synthesis</td>
</tr>
<tr>
<td>Glutamate Cysteine Ligase Modifier (GCLM)</td>
<td></td>
</tr>
<tr>
<td>NAD(P)H:quinone oxidoreductase (NQO1)</td>
<td>NAD(P)H + Sub (\rightarrow) NAD(P)\textsuperscript{+} + Sub-H</td>
</tr>
<tr>
<td>Peroxiredoxin (Prx)</td>
<td>2 Prx-H + H\textsubscript{2}O\textsubscript{2} (\rightarrow) 2 H\textsubscript{2}O</td>
</tr>
<tr>
<td>Sulfiredoxin (Srx)</td>
<td>Makes disulfide bonds using Prx &amp; ATP</td>
</tr>
<tr>
<td></td>
<td>Ex: Prx + 2 R-SH (\rightarrow) Prx-H + R-S-S-R</td>
</tr>
<tr>
<td>Thioredoxin (Trx)</td>
<td>Replenish peroxiredoxin</td>
</tr>
<tr>
<td></td>
<td>Ex: Trx-H + Prx (\rightarrow) Trx + Prx-H</td>
</tr>
<tr>
<td>Thioredoxin reductase (TrxR)</td>
<td>Replenish Trx-H via NADH</td>
</tr>
<tr>
<td></td>
<td>Ex: Trx + NADH (\rightarrow) Trx-H + NAD\textsuperscript{+}</td>
</tr>
<tr>
<td>G6PD</td>
<td>Pentose phosphate pathway enzymes; PPP creates NADH</td>
</tr>
<tr>
<td>PGD</td>
<td>Citric acid cycle enzyme, creates NADH</td>
</tr>
</tbody>
</table>

The overall influence of the expression of aforementioned Nrf2-inducible genes is expansive and is depicted nicely by this figure below from a 2009 review article on Nrf2’s role in inflammatory injury.\textsuperscript{117} Reviewing each portion of the flow chart is beyond the scope of the present review, as we will remain focused on ROS-mediated damage to cardiac myocytes in the early stages of MI ischaemia-reperfusion. Taken as a whole, the impact of Nrf2 is clearly anti-inflammatory, metabolically stabilizing, and cytoprotective.
Keap1 Regulation of Nrf2

The Yamamoto group, who discovered that Nrf2 bound to ARE and upregulated cytoprotective gene expression in 1997, were also the first to publish on what is now considered the ‘canonical’ mechanism of negative regulation of Nrf2. In 1999, it was documented that electrophilic stress in macrophages caused an increase in Nrf2 DNA binding, yet Nrf2 mRNA levels remained unchanged. This finding indicated that Nrf2 activity may be regulated at the protein level and that electrophilic stress transduced a signal that caused Nrf2 to translocate from the cytoplasm to the nucleus. After selectively deleting various Neh domains of Nrf2, the N-terminal Neh2 domain was shown to be required for its negative regulation. Using a two-hybrid yeast system of Gal4-Neh2 as bait and a 17 days post-conception mouse embryo cDNA library as the prey, the protein causing this negative regulation could be “fished out” using this bait and prey method that enabled yeast clones to grow in histidine-deficient medium. These clones were isolated and revealed a single protein bound to the Neh2 domain of Nrf2. Inspection of the protein’s sequence revealed two noteworthy motifs – a BTB domain and a double glycine repeat (DGR) domain – a combination of motifs typical of the *Drosophila* actin-binding protein, Kelch. The authors named their newly discovered protein *Keelch-like ECH (Nrf2) associated protein-1* (Keap1).
Again using the Neh2 domain of Nrf2 in the two-hybrid yeast model, investigators selectively baited either the BTB or DGR motifs of Keap1 to further explore the Keap1-Nrf2 protein-protein interaction, with results showing the DGR motif is responsible for binding to Nrf2. Using luciferase-tagged Nrf2 in the presence of GFP-Keap1, it was exhibited that electrophilic stress increased Nrf2 protein presence in multiple cell types. Finally, colocalization studies using immunohistochemistry indicated that Keap1 sequestered Nrf2 in the cytoplasm, thus inhibiting Nrf2’s action as a transcription factor, and electrophilic stress resulted in Keap1-dissociated Nrf2 that could translocate to the nucleus. Taken together, this data confirmed the notion that Keap1-Nrf2 acts a cytoplasmic sensor of electrophilic (oxidative) stress, and under basal conditions, Keap1 precludes Nrf2 from acting as a transcription factor by virtue of cytoplasmic sequestration. In 2002, Zipper & Mulcahy contributed to the collective understanding of how Keap1 exerts this effect on Nrf2 by showing that Keap1 must homodimerize through its BTB domain to keep Nrf2 in the cytoplasm. Further investigation of the Keap1-Nrf2 interaction was warranted to determine if there was anything more to the negative regulation of Nrf2 than merely physical sequestration.

Evidence that Nrf2 is turned over through Keap1-dependent ubiquitin-mediated degradation via the 26s proteasome was shown in vitro by multiple investigators in the early 2000-oughts. Yamamoto and collaborators determined that the Neh2 domain of Nrf2 was the degron, with one of the seven lysine residues in that domain being the target for polyubiquitination under basal (reducing intracellular environment) conditions.

It was shown soon after by Zhang & Hannink in 2003 that two cysteine residues on Keap1 are critical for Keap1-dependent ubiquitination of Nrf2 in the Keap1-Nrf2 complex. They proposed Keap1 is part of an E3 ubiquitin ligase complex that is regulated by oxidative stress and chemopreventive agents. A basic review on the process of ubiquitin-mediated proteasomal degradation of proteins is depicted below:
Figure 29: Ubiquitin-proteasome system at a glance. Ubiquitin (Ub) activating enzyme (E1) uses ATP to bind Ub, which is then transferred to ubiquitin-conjugating enzyme (E2). Ubiquitin ligases (E3) confer specificity in this system, as they can only bind to select proteins. E3-protein complex can associate with E2-Ub, resulting in ubiquitination of the substrate protein on various lysine residues. Polyubiquitin chains of (four or more; not shown) on the substrate protein tag it for degradation via the 26s proteasome.

Keap1-mediated regulation of Nrf2 requires reducing conditions, i.e. is disrupted in oxidative conditions, and thiol-reactive chemicals or their metabolites such as t-BHQ or sulforophane cause Nrf2 accumulation and Nrf2-mediated induction of ARE-associated genes. Zhang & Hannink set out to determine which, if any, specific cysteine residues within Keap1 serve as reactive redox sensors of oxidative environment and repress ubiquitination of Nrf2. They created seven distinct full-length Keap1 protein constructs with single cysteine to serine residue mutations, four in the BTB domain (perhaps based on Zipper & Mulcahy’s evidence of BTB dimerization being essential for Keap1 sequestration of Nrf2, though not explicitly stated in their publication) and three in the Linker domain based on evidence published by Yamamoto and collaborators in 2002. C273 and C288—both in the Linker domain of Keap1—proved to be essential for the negative regulation of Nrf2 under basal conditions, evidenced by a half-life increase of ectopic HA-tagged Nrf2 from ~40 minutes in the presence of WT Keap1 to over 6 hours in the presence of Keap1-C273S. These mutations did not perturb the ability of Keap1 to bind to Nrf2 but rather the Keap1-mediated ubiquitination of Nrf2, indicating that Nrf2 was stabilized.

C151S mutation (located in the BTB domain of Keap1), on the other hand, resulted in constitutive repression of Nrf2 by Keap1 regardless of the oxidative
climate, causing loss of the Nrf2 response to chemopreventive compounds; t-BHQ or sulforophane treatments in the presence of C151S Keap1 had no impact on HA-Nrf2 half-life times of ~40 minutes on pulse chase experiments, whereas in the presence of WT Keap1, t-BHQ or sulforophane treatment resulted in a HA-Nrf2 half-life increase to >150 minutes. This means that C151 of Keap1 is essential for appropriate redox sensitivity of the Keap1-Nrf2 regulatory system.

Zhang et al then published that Keap1 serves a substrate adaptor in an E3 ubiquitin ligase complex specifically with the scaffolding proteins Cullin 3 (Cul3) and Ring box protein 1 (Rbx1), the latter of which has E2 protein binding, which brings ubiquitin in very close proximity to the Nrf2 substrate. Interestingly, Nrf2 inducers such as t-BHQ and sulforophane do not cause physical dissociation of the Keap1-Nrf2 complex, but rather stabilize existing complexes by inhibiting ubiquitination of Nrf2.

In 2006, it was determined that Keap1 homodimerizes through both BTB domains and interacts with Cul3. This leaves two Kelch-like domains of Keap1 free to interact with the DLG and ETGE domains of Nrf2. The Keap1 association is much stronger (200-fold greater) with the ETGE motif than with the DLG motif of Nrf2. The disparity in protein-protein interaction between the two motifs has been described as a “hinge and latch” model, much like a door where one end is anchored to Keap1 (the ETGE motif of Nrf2 acting as the ‘hinge’) and the other end is more readily opened and closed (the DLG motif of Nrf2 acting as the ‘latch’). Binding both ETGE and DLG domains of Nrf2 are essential for Keap1-mediated ubiquitination as it induces a conformation of Nrf2 within the E3 ubiquitin ligase complex that allows Nrf2 to be polyubiquitinated. Keap1 contains 27 cysteine residues that can react covalently, oftentimes with electrophiles, and these modifications are believed (based on selective deletion experiments such as Zhang & Hannink’s C151S substitution previously described resulting in loss of redox-sensitivity of the Keap1-Nrf2 complex and additional research by Yamamoto and colleagues to induce a conformational change such that Keap1 and the DLG motif of Nrf2 dissociate, rendering Nrf2 unable to be ubiquitinated and stabilizing the complex. Keap1-Cul3-Rbx1 complexes become saturated with stable Nrf2 and newly synthesized unbound Nrf2 protein accumulates in the cytoplasm, able to translocate to the nucleus and work as a transcription factor.
Assimilating all of these findings together, we arrive at the canonical mechanism of Nrf2 regulation, pictured below.

**Figure 30:** Structure summary of Nrf2 and Keap1 domains/key features.

As described by a 2013 review by Jaramillo & Zhang, "Under basal conditions, Keap1 binds to the ETGE and DLG motifs on Nrf2 and brings Nrf2 into Keap1–Cul3–E3 ubiquitin ligase complex, leading to ubiquitination and subsequent degradation of Nrf2. Oxidative stress or electrophiles can cause a conformational change in the Keap1–Cul3–E3 ubiquitin ligase by acting on specific cysteine residues in Keap1. These changes disrupt Nrf2–Keap1 binding at the DLG domain. Nrf2 is stabilized, and free Nrf2 translocates to the nucleus, where it dimerizes with members of the small Maf family and binds to AREs (5′-RTGABNNNGCR-3′) within regulatory regions of a wide variety of cell defense genes, including NQO1, GCLM, HO-1, and MRP1. (E) ETGE; (D) DLG."

**Figure 31:** Nrf2 regulation via Keap1, the canonical pathway.

Alternative Mechanisms of Nrf2 Regulation

The multi-faceted cytoprotective gene profile induced by Nrf2 appears to be something of a golden goose in the context of myocardial infarction or, say, neurodegenerative diseases. However, on the other side of the coin is the tremendous benefit that dysfunctional Nrf2 regulation could confer to cancer cells. Oncogenic mutations or signaling that would result in constitutive Nrf2 activity would give cancer cells an upper hand against many of the cancer
therapies of the day. This has given Nrf2 a reputation for having a “dark side.” Alternative mechanisms of Nrf2 regulation are likely of more immediate interest to those wishing to diminish its activity, as opposed to the goal of this review. However, for completeness, some of the established alternative methods to Keap1 regulation will be reviewed.

Nrf2 is the target of two other E3 ubiquitin ligases besides Keap1, though aforementioned experiments specifically involving Keap1 are evidence that the regulatory contribution of either of these two E3 ligases is not as substantial. One such E3 ligase is β-transducin repeat-containing protein, β-TrCP. One of the two β-TrCP binding sites in Nrf2’s Neh6 domain can be targeted by glycogen synthase kinase-3 (GSK-3), which increases β-TrCP affinity and therefore increases degradation. The phosphatidylinositol-3-kinases (PI3K), which are signal transducers of G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs), phosphorylates (activates) AKT, which in turn phosphorylates (in this case, inhibiting) GSK-3. PI3K and AKT inhibitors sensitize Keap1/- mice to redox stress, and this effect is reversed by GSK-3 inhibition.134 The other E3 ubiquitin ligase that regulates Nrf2 is Hrd1, which is anchored to the endoplasmic reticulum and involved in ER stress and the XBP-1-associated arm of the unfolded protein response (UPR).135,136 It is counter-intuitive that an E3 ligase associated with a (ER) stress response would negatively regulate Nrf2. The UPR is indeed activated as a result of MI.137 This makes determining the extent that Hrd1 hinders Nrf2 signaling in the context of myocardial infarction and post-MI remodeling an exciting new line of questioning with the potential to yield a new therapeutic target.

The process of autophagy, large-scale turnover of cellular components (from bulk protein turnover to entire organelle turnover) – typically a response to metabolic insufficiency – is also implicated in Nrf2 regulation. Autophagy is a process that occurs to some extent in normal cardiomyocytes but is increased during stress. One review proffered a “Goldilocks rule” for autophagic flux in that both too much and too little autophagic activity are indicative and/or contribute to different forms of heart disease.138 The protein p62 destines cargo (other proteins) for the autophagosome upon binding. P62 has a domain resembling the ETGE domain of Nrf2, and can bind Keap1, thus targeting it to the autophagosome and removing negative regulation of Nrf2.139 Our review of cardiomyocyte physiology illustrated their voracity even under normal conditions, and autophagy is activated as a result of myocardial infarction in an attempt to meet these steep energetic demands. In a permanent LAD occlusion mouse model, inhibition of autophagy increased infarct size by 31%. Pre-ischemic starvation was associated with increased autophagy and a protective effect (23% infarct size reduction).140 Autophagic activity decreases with age141 and acute myocardial infarctions typically occur in middle-aged to elderly individuals, making this mechanism of Nrf2 activation yet another possible angle to explore.
**Exercise, Ischemic Pre-conditioning, and Nrf2**

It is important to distinguish between the environment cardiomyocytes experience as a result of exercise, with bouts of hypoxia, and ischemia-IRI secondary to prolonged infarction and reperfusion in acute MI. The former, even after short-term regimens, elicits protective effects to preclude or at least limit damage from the latter. Studies using Sprague-Dawley rats have shown that to gain myocardial-protective benefits from exercise, a minimum threshold of intensity (percentage of VO$_{2\text{max}}$) must be met, but there is evidence indicating no linear benefit beyond the minimum requirement; moderate exercise (55% VO$_{2\text{max}}$) is sufficient and as beneficial as high-intensity exercise (75% VO$_{2\text{max}}$). Current clinical counseling and guidelines from U.S. Department of Health and Human Services are in line with this. Aged rats exhibit decreased cardiac SOD and Hsp70 - both of which are cytoprotective - compared to younger counterparts, but both age-related changes are attenuated with moderate exercise training. Furthermore, aged rats exhibit increased cardiac apoptosis (increased Bax/Bcl-2 ratio), which is also attenuated with moderate exercise. Nrf2 happens to mediate, at least in part, all three of these molecular entities; Nrf2 binds to AREs upstream of the SOD gene, Hsp70 gene, and the anti-apoptotic Bcl-2 gene. Nrf2 signaling itself is decreased in aged mice, but this, too, is restored following moderate exercise training. Because Nrf2 precedes (i.e. can increase transcription of) the three previously mentioned molecular entities spanning oxidative stress, protein chaperoning, and apoptosis, when one considers exercise as a form of medicine, it is clear that many effects are mediated at least in part through Nrf2, although there can be redundancy in cell signaling, exemplified by the transcription factor Heat Shock Factor 1 (Hsf1) also inducing Hsp70 expression. A recent (2016) review elaborating on the modest oxidative burden created by exercise and the essential role Nrf2 signaling for subsequent cytoprotection can be found here.

**Discussion**

This review detailed ischemic impact on ion gradients leading up to mitochondrial calcium overload, mitochondrial depolarization (priming MPTP opening), and disruption of the ETC such that it generates ROS that damage cardiolipin, becomes uncoupled, and is then primed to act as an ROS-generating machine upon reperfusion. In this way, calcium dysregulation and ROS potentiate one another. Calcium channel blockers (CCBs) already exist and are used in the clinic, and while the rationale is clear for this methodology and our review could breathe fresh life into the discussion, this is not a review on CCBs. We channel our focus here on providing background and context to encourage further research and clinical applications centered on dampening oxidative stress (which happens to occur in large part as a result of mitochondrial calcium overloading). Given any additional buffering capacity against oxidative stress, essential components of
the mitochondria such as cardiolipin and the complexes it serves as an essential scaffold for would likely retain their ‘intended’ physiological function. The bioenergetic failure and ROS burden experienced during early stages of acute MI could be attenuated -- to what extent remains to be seen, but despite how far we have come, all signs point to there being room for significant further improvement in the treatment of these acute coronary events.

The ultimate size of infarcts is directly related to the length of time the tissue is subjected to ischemia.\textsuperscript{174}

\textbf{Figure 32:} Using radioactive microspheres in a dog myocardial infarction model, collateral arterial flow was measured after 10 minutes of ischemia. Areas of high collateral flow exhibit a slower rate of necrosis, but necrosis nonetheless occurs as a direct function of length of ischemia.\textsuperscript{174}

A 2011 study integrated data from animal models, cardiac MRI in humans, and clinical outcomes (lives saved) and concluded that all follow the same trend line with respect to ischemia. Limiting ischemia to <120 minutes confers the most benefit.\textsuperscript{173}
Figure 33: Animal modeling extent of infarct (green triangle), human cardiac MRI infarct assessment (red square), and clinical outcomes (blue diamond) of myocardial infarction all follow a similar trend with respect to length of time of ischemia.\textsuperscript{173}

While the ischemia-cell death relationship seems straightforward and the conclusion that follows is to reperfuse the ischemic tissue as quickly as possible, we see a phenomenon where the very solution to the problem, reperfusion, causes a stark increase of cell death – even moreso than if the tissue were simply left ischemic (see figure below).\textsuperscript{179}

![Graph showing cell death percentage over time with and without reperfusion.]

**Figure 34:** Chick cardiomyocytes subjected to ischemia-reperfusion exhibit far more cell death than cells simply left ischemic over a four-hour period (50% death compared to 10%, assessed by uptake of propidium iodide).\textsuperscript{179}

In the long run, of course, tissues left ischemic will virtually all die, so it is still best to opt for reperfusion. Yet the extent of cell death resulting from reperfusion seems egregious and presents the scientific community with a new challenge.

Putting these events together, we see that the physiological induction of Nrf2 is evidently "catching up" to insults; it evades Keap1-mediated regulation and goes on to increase transcription of cytoprotective genes after the fact. When cellular changes occur as rapidly as they do in the context of acute MI, cells are essentially fighting a losing battle, as evidenced by pathohistological findings following reperfusion. Means of pre-emptively activating Nrf2 transcriptional activity and subsequently boosting Nrf2-inducible enzymes to buffer/prepare for IRI is a strategy that has yet to be fully explored in the laboratory, let alone tested/implemented in the clinic.
Gradual reintroduction of oxygen during reperfusion would, in principle, allot cells time to acclimate and allow antioxidant defenses (virtually all of which can be induced by Nrf2) to “catch up.” When put into practice these principles yield results that could change reperfusion protocols throughout the world. In a rabbit model, postconditioning inhibits MPTP opening and limits infarct size to 29% of the at-risk region, as opposed to 61% infarct size of the at-risk region when reperfusion occurred suddenly. In humans, 30 second cycles between opening/closing of the artery (i.e. postconditioning) for the first three minutes of PPCI reduced infarct size from 31% to 23%. Another study in humans found reduction of no-reflow phenomenon, an adverse effect that clinicians have had no course of action against in the past. With proper perfusion came a reduction in infarct size and better ventricular ejection fraction (55% vs. 43%). These are major improvements on an already life-saving procedure, and the effort and cost required to promote these effects appear somewhat trivial (opening and closing the artery in a cyclic fashion over the course of a few minutes). 30 second cycles may seem mundane, but for an enzyme such as catalase, which has a kinetic activity close to “kinetic perfection” i.e. catalyzes $\text{H}_2\text{O}_2 \rightarrow \text{O}_2$ as rapidly as $\text{H}_2\text{O}_2$ can diffuse, these are critical moments in determining the fate of a cell.
present review implores: what if, by way of therapeutic Nrf2 induction, there was more catalase in the first place?

Pre-conditioning (i.e. exercise) appears to exhibit an even greater protective effect than postconditioning. However, we do not have the luxury of knowing when an acute coronary event will occur, so whether there will be benefits of pre-conditioning is in many ways a matter of chance. The same applies to the phenomenon of remote pre-conditioning, whereby transient, purposely induced ischemia in an unrelated area (such as the leg) prior to cardiac ischemia/reperfusion can attenuate cardiac cell death. Remote post-conditioning, which was worth trying but seemed to be a bit of a leap of faith, does not appear to confer any benefit.

Just as exercise induces Nrf2 activity, so too do many foods that are generally considered to be part of a balanced diet and "good" for us. Broccoli and Brussels sprouts contain sulforaphane, a prototypical Nrf2 inducer. Rather than go the high throughput screening route of drug discovery for a Nrf2-activating compound, many researchers are indicating that nature may have already done the job for us. Administration of niacin, a NAD precursor, provided cardioprotection from IRI in isolated rat hearts. Another study showed niacin staved off vascular inflammation following unilateral carotid artery ligation in rabbits – the protective effects being attributed to Nrf2 by way of HO-1 induction. Mangiferin, a compound extracted from the mango plant, increases Nrf2’s protein half-life from 20 minutes to 58 minutes by inhibiting ubiquitination of Nrf2 in hematopoietic cells. There is a patented proprietary blend of Nrf2-activating phytochemicals under the name Protandim which has been vetted in scientific journals and proven to increase nuclear Nrf2 and protect against H₂O₂-induced apoptosis in human coronary artery endothelial cells (HCAECs). It will be exciting to see what the results are if and when the product is taken to the organism level and/or after some time in the market in the context of diet, exercise, age, and of course myocardial infarction. Based on these collective findings, a very strong case can be made for using Nrf2-inducing compounds in the clinic as adjuvant therapies to PPCI and/or thrombolytic acute MI reperfusion therapies in order to reduce the ROS burden and therefore reduce cellular dysfunction and death.

Statin drugs have been shown to activate Nrf2 via PI3K, a discovery made by Joshua Strom, PhD of the University of Arizona while completing his dissertation in 2014. These drugs were developed to lower cholesterol, so the Nrf2-activating component of many different statin compounds (atorvastatin, simvastatin, lovastatin, pravastatin) may be a fortuitous -- though unintended -- additional activity. Perhaps this is a factor in the distinction between how well chronic statin users fare compared to their non-statin-taking counterparts following a myocardial infarction. For example, grade 3 TIMI flow (normal reperfusion) following PPCI was achieved in 95% of chronic statin users compared to 83.5% of the nonstatin group. Additionally, Nrf2 activation
through statin drugs may be a contributing factor to the reduced in-hospital mortality observed when patients are started on early-statin therapy following acute MI. Benefits are also observed in patients with acute MI even when they have ideal “bad” LDL cholesterol levels (<70mg/dl; the clinical target value). 2016 recommendations from the US Preventive Services Task Force published in the Journal of the American Medical Association broaden the indications for statin therapy beyond cholesterol and lipid levels. As the specific downstream effects of PI3K activation on Nrf2 have yet to be elucidated, more research on that front may be warranted. Furthermore, as the PI3K-based activation is almost certainly rooted in phosphorylation (of Nrf2), that leaves other means of intervention on Nrf2 regulation available and currently unexplored as therapeutic targets.

In macrophages and bronchial epithelial cells grown in culture, exposure to diesel exhaust fumes causes increases in Nrf2-inducible proteins such as HO-1, SOD, and GST after 6 hours. Interestingly, this stressor does not cause increases of Nrf2-inducible proteins equally; catalase and NQO1 were not increased at all. Perhaps this can be attributed to the cell types used in the study or that there is nuance in what profile of genes are expressed following exposure to various Nrf2-inducing chemicals despite the common denominator of Nrf2. Either way, it is tangential to our topic at hand and will not be explored further here, though it will be worthwhile to verify which ARE gene “profile” is induced by which compounds if pharmacotherapies in the context of acute MI are pursued in the future, as they are likely not all equal (for instance, an entire review published in 2015 was devoted to how sulforophane specifically activates Nrf2).

In mouse cardiac fibroblasts exposed to D3T (a chemical inducer of Nrf2), mRNA levels of Nrf2-inducible genes increase as early as three hours, peak at 24 hours, and remain elevated as far out as 48 hours. In a 90-minute balloon myocardial infarction model in dogs, both infarct size and extent of microvascular obstruction increases over a 48-hour period following reperfusion. Taken together, this means that there should theoretically still be substantial benefit in upregulating Nrf2 even after onset of acute MI, as the condition progresses and spreads for up to 1 week (based on contrast-enhanced cardiac MRI findings) but cytoprotective genes can be induced in neighboring at-risk tissue in as little as 3 hours.
In cardiac fibroblasts \textit{in vitro}, Nrf2-dependent increases in NQO1 mRNA are observed as early as three hours after exposure to 100uM 3H-1,2-dithiole-3-thione (D3T), a chemical inducer of Nrf2. Expression continues to increase and remains >10x higher than baseline for at least two days.\textsuperscript{169}

In the cells immediately affected by ischemia-reperfusion, Nrf2 is induced physiologically within 10 minutes, so the extent of benefit from exogenous upregulation of Nrf2 in these cells is less clear.\textsuperscript{171} Neighboring cells subjected to latent inflammation and microvascular obstruction as the infarct spreads\textsuperscript{173} may benefit most from Nrf2-upregulating therapeutic strategies. Ideally, exogenous Nrf2 upregulation would be administered before an infarct ever occurs, but clinicians and patients do not have the luxury of knowing when that will be. Timing and access to patients who have an acute MI in versus outside of a hospital is starkly different; a study published in 2000 assessing ~6,000 patients with MI showed the average interval from symptom onset to intervention was 55 minutes for in-hospital cases and 180 minutes for out-of-hospital cases ($p=0.001$).\textsuperscript{188} In-hospital cases only made up a small subset (~7%) of the acute MI cases in the study. Despite more rapid treatment time, mortality rates for in-hospital cases versus out-of-hospital cases were 27% and 14%, respectively. Authors attributed this to in-hospital patients tending to be older and sicker, with higher prevalence of concomitant diseases such as HTN, type II diabetes, renal insufficiency, or contraindications for thrombolysis.\textsuperscript{188}

Perhaps a subset of (currently hypothetical) Nrf2 therapies could be developed with a delivery rationale similar to oral nitroglycerin taken by patients with unstable angina to address the issue of timing. Furthermore, as nitroglycerin causes vasodilation, if Nrf2 therapies are taken concurrently with nitrates they could reach the intended tissue and help at-risk cells buffer/prepare for impending infarcts before they happen. While it is an exciting prospect the author hopes will encourage further research, it is admittedly only hypothetical at the time of this writing.
One important issue to address would be how best to deliver a Nrf2-activating compound to the intended target tissue. The author proposes that using a local drug delivery catheter during PPCI or a drug-eluting stent are both relatively obvious ideas. Nanoliposomes as drug delivery vehicles are a relatively novel delivery method, and can likely confer specificity because their composition is at our discretion. The author posits that perhaps constructing nanoliposomes that would recognize and bind to tissue factor would localize them to sites of compromised endothelium such as atheroma rupture sites. Of course, infarcts occur downstream of atheroma ruptures (if atheroma rupture is indeed the cause), so perhaps a heterogenous nanoliposome mixture could be administered, with a percentage constructed to bind to distinct features of endothelial microparticles (EMPs). EMPs can cause microvascular obstruction in tributary capillaries of an infarcted artery. If/when methods of determining root cause of the infarct develop to make rapid distinctions prior to arriving at the catheter lab, it is not difficult to imagine there being a spectrum of Nrf2-activating therapeutic options to choose from and tailor to each patient case. Whatever the iteration may be, data from research labs all around the globe indicate that Nrf2-directed therapies in MI seem extremely promising. Improvements to reperfusion methods and management of acute MI, whether direct or indirect, will almost certainly go through Nrf2, as it has a sweeping presence and is at the crux of the cellular stress response.

There are admittedly some elephants in the room when one considers that apoptosis, autophagy, and numerous cellular pathways are also at play in the context presented in this review (MI). The present article is looking at the beginning of the acute event -- a logical first step -- and weighing the pros and cons of Nrf2 as a candidate therapeutic target in AMI. The author looks forward to exploring and reviewing additional modes/mechanisms of myocardial damage and death in the context of Nrf2 in the future.

In terms of negative effects of Nrf2, a “dark side” to the master regulator of cellular stress has been acknowledged, but it is more applicable to cancer than it is to MI. Constituitive Nrf2 activity in cancer cells can confer many benefits, including chemoresistance, and contribute to the tenacity of this particular disease. In the context of myocardial infarction, transient Nrf2 activation seems to be virtually free of negative consequence.

Let us take a moment to pause and reflect that much of this damage and cell death was initially sparked by a modest increase in ROS due to ischemia. In conditions suited for a perfect storm, a modest burden of ROS is able to induce a ripple effect with the potential to build to a wave of gross transmural necrosis. These cellular events affect millions of people every year and are the basis for the number one cause of death in the world. What if the cells in question had more antioxidant defenses mounted -- would infarct and peri-infarct cells stand a better chance? What if a battery of antioxidant defense genes could be induced by a single transcription factor? The basic science behind this transcription factor
seems to have been largely elucidated, and the rationales of implementation are vetted in publications from labs all around the world. It is with much optimism and excitement that the author of this review asks: to what extent could the impact of myocardial infarction be attenuated if we harness the power of this “master regulator of stress,” Nrf2, in the clinic?
References

18. Quinaglia e Silva JC, et al. Peri-infarct zone characterized by cardiac magnetic resonance imaging is directly associated with the inflammatory activity during acute phase myocardial infarction. *Inflammation* 2014;37(3):678-85.


101. Chowdry S, et al. Nrf2 is controlled by two distinct β-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity. *Oncogene* 2013;32(32):3765-81.


121. Sekhar KR, et al. Nrf2 degradation by the ubiquitin proteasome pathway is inhibited by KIAA0132, the human homolog to INrf2. *Oncogene* 2002;21(44):6829-34.


134. Chowdry S, et al. Nrf2 is controlled by two distinct β-TrCP recognition motifs in its Neh6 domain, one of which can be modulated by GSK-3 activity. *Oncogene* 2013;32(32):3765-81.


