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Leveraging oxidative stress questions in vivo: Implications and limitations

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Abstract

The elegance of Helmut Sies' original definition of oxidative stress belies the complexity of the reactions that are potentially involved. This is by no means a criticism of the author, but rather how the words have been used to oversimplify the concept by some. Reactive oxygen and nitrogen species (ROS and RNS, respectively) can be products of a myriad of events within the living body. Indeed, it is now understood that ROS/RNS are critical for normal cellular metabolism and have beneficial effects (e.g., cytotoxicity against invading bacteria). A general problem of studying prooxidants in vivo is that, due to their inherent reactivity, they generally cannot be measured directly. This indirect detection of 'footprints' leaves a very large black box that we are to this day only beginning to understand. This manuscript will summarize some considerations that are of utmost importance when translating oxidative stress into in vivo research. Helmut has been a key thought leader, researcher and mentor whose contributions to this field are immeasurable.

1. Introduction

Originally, it was generally thought that free radical formation in the cell was limited to either external stimuli (such as radiation), or random events, and did not occur under "normal" circumstances. A major paradigm shift in free radical research came in 1969, when Fridovich and McCord described the function of SOD as a catalytic reducer of O_2^- to H_2O_2 [1]. Based on these results, the concept that oxidants are produced by the cell under normal conditions gained hold. The discovery of enzymes that normally produce prooxidants (in contrast to electron leakage from other enzyme systems), such as NAD(P)H oxidases, xanthine oxidases, and myeloperoxidases, further strengthened the case that prooxidants are regularly found in vivo. Discoveries of catalytic functions of the glutathione peroxidases, glutaredoxin peroxidases, thioredoxin peroxidases, and catalases indicated that hydroperoxides are also kept in close check in vivo. It became clear that balance between prooxidants and antioxidants is critical for survival and functioning of aerobic organisms. An imbalance favoring prooxidants and/or disfavoring antioxidants, potentially leading to damage, was coined as oxidative stress by Helmut Sies [2].

2. How I met Helmut

My dissertation focused on developing a biochemical marker for hepatic hypoxia. A gradient of oxygen exists in liver lobule [3], which makes it exquisitely prone to hypoxia in

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downstream regions. It had been proposed in the early 1970's that ethanol consumption causes hepatic hypoxia [4]. However, the "hypoxia hypothesis" of alcohol-induced liver injury was highly controversial, with several studies supporting or refuting it (e.g., [5,6]). This controversy was fueled, at least in part, by the technical limitations of measuring hepatic oxygen tension at the cellular level in intact animals. We employed a hypoxia marker, called pimonidazole, which was initially developed to detect hypoxia in solid tumors [7]. This 2-nitroimidazole initially undergoes a single electron reduction; the resulting nitro radical anion is reoxidized to the parent molecule by molecular oxygen. In cells that are hypoxic, further reduction proceeds and the resultant thiol adduct can be detected immunochemically [8]. This approach was used to demonstrate that alcohol consumption indeed causes hepatic hypoxia [9,10], which is now an accepted mechanism by which alcohol damages the liver and has led to the development of new 'druggable targets' for ALD [11].

My dissertation research brought Helmut's work to light for me. First, he published seminal work demonstrating that cells have steep intracellular oxygen gradients [12], which was critical for justifying the invalidity of using blood oxygen tension to determine intracellular oxygen concentrations. Second, the impact of hepatic hypoxia on oxidative stress was a topic on which I focussed during the end of my dissertation; this research sparked my interest in peroxynitrite as a potential ROS/RNS in the liver [13]. It was in this timeframe that Helmut's group was describing a new function of selenoproteins (and organoselenium compounds) as peroxynitrite reductases [14]. I had learned enough about oxidative stress during my dissertation work to know that it interested me, but that I only had a superficial knowledge of the field. I decided that I wanted to gain more in depth understanding of oxidative stress during my post-doctoral training.

Helmut and my doctoral mentor (the late Ron Thurman) knew each other well both from their mutual time in Munich, as well as through work with Britton Chance at the Johnson Research Foundation. Ron spoke very highly of Helmut and it seemed an ideal match for a postdoctoral training experience. I contacted Helmut and visited Düsseldorf for a week (Fig. 1). We wrote an *Alexander von Humboldt* fellowship together and I moved to Germany in the beginning of 1998. It should be noted that I didn't know a word of German at that time (Fig. 2).

3. The Sies years (1998–2000)

The major focus of my research with Helmut was to continue to characterize the direct and indirect functions of selenoproteins as peroxynitrite reductases (e.g., [15,16]). We also investigated the interaction of catechin oligomers and organotellurium compounds with peroxynitrite and/or it's degradation products [17–19]. Helmut also involved me in some interesting (and challenging) side projects, such as characterizing the heparin-binding properties of selenoprotein P via surface plasmon resonance [20]. This latter project suggested that selenoprotein P potentially functions as protective 'coating' on the glycocalyx, a function similar to what is hypothesized for extracellular superoxide dismutase [21]. We also wrote several reviews together, one of which is still cited on a frequent basis [22].

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As mentioned above, the other scientific goal of my postdoctoral training was to learn more about oxidative stress biochemistry and antioxidants. Immersion into Helmut's group was an ideal oppurtunity to increase my knowledge base about oxidative stress. Just trying to get up to speed on what was considered 'background' in that group was a humbling experience. Helmut practiced a good balance of directed independence that I try and emulate in my training now. I found the research compelling and fun, to the point that to this day I periodically whistfully entertain the notion of returning to free radical reaction chemistry in my group. This experience also indirectly taught me that it is relatively easy as a scientist to change foci and embark on new areas/lines of research; I believe that this understanding is critical for young scientists.

In addition to the scientific experience, Helmut afforded me oppurtunities that dramatically improved my preparedness for a career as an independent scientist. Mostly notably, he promoted me to "*Arbeitsgruppenleiter*" in 1999; Helmut's Institut was very large and was broken up in smaller research teams (*Arbeitsgruppen*) with related research themes. I unlearned the myopic view of a research trainee and began the process of thinking about other researchers and projects in the group as a supervisor. I have always been thankful for this experience, as it made transitioning to a to a PI later in my career a much less painful process. Helmut offered for me to stay longer in Germany, which was very tempting. However, my long-term goal was to attain a faculty position in the US and I felt I needed to get to work on that goal. Thus, at the end of my two year fellowship, it was with strongly mixed feelings that I returned to the States.

4. Once a *Siesianer*, always a *Siesianer*

I returned to in vivo hepatobiology at the beginning of 2000. Some of the work during that time was collaborative with Helmut, investigating the role of oxidative stress in alcoholic liver disease (e.g., [23,24]), which was an outstanding bridge between the two fields. I began to understand that there was potentially a niche for me to fill in the liver field. Whereas there are several outstanding free radical chemists/biochemists out there, and equally as many hepatologists, there were only a handful of individuals that could span both fields successfully. Indeed, my impression was that most in vivo toxicologists viewed oxidative stress as an endpoint in their experiments rather than as a mechanistic means. I applied the training I received under Helmut to try and ask such mechanistic questions, especially in the field of alcoholic liver injury.

Our work demonstrating that iNOS knockout mice are almost completely protected against alcoholic liver injury serves as such an example to this point [25]. At the time this manuscript was published, the prevailing hypothesis in the field was that $NO^{\bullet}$ played a protective role in alcoholic liver disease [26]. The concept that $NO^{\bullet}$ production was both protective (via eNOS to maintain hepatic vascular tone) and damaging (via iNOS to produce RNS) was viewed as novel. This was a critical time in my career progression when I was transitioning to true independence and "being known" for something was highly beneficial. In this time-frame, I was asked to write a review article on the subject of oxidative stress in alcoholic liver disease [27]. I credit Helmut's training for the concepts that I put down in that review, as well as for putting me on the path that gave the chance to write it.

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I continue to “follow my nose” (homage to J. Michael Bishop intended) in research. A major focus of our current work is understanding the role of transitional matrix remodeling in hepatic inflammation. Although oxidative stress is not as prevalent a facet of the work of our group, it is still never very far from our thoughts and it is still a critical mechanistic foundation upon which the research builds. More importantly, Helmut’s approach to science has always been “vertically integrated” rather than “horizontally diffuse.” Even on projects that have nothing to do with oxidative stress, his training influences my experimental approach. I will always be a proud protégé of Helmut … a *Siesianer*.

5. Oxidative stress in vivo: implications and limitations

The elegance of Helmut’s original definition of oxidative stress [2] belies the complexity of the reactions that are potentially involved. This is by no means a criticism of the author, but rather how the words have been used to oversimplify the concept by some. Reactive oxygen and nitrogen species (ROS and RNS, respectively) can be products of a myriad of events within the living body. Indeed, it is now understood that ROS/RNS are critical for normal cellular metabolism and have beneficial effects (e.g., cytotoxicity against invading bacteria); indeed, there are enzymes in which the key function is to produce ROS/RNS, such as nitric oxide synthases (NOS), NA(D)PH oxidases, and myeloperoxidases. A general problem of studying prooxidants in vivo is that, due to their inherent reactivity, they generally cannot be measured directly. One is therefore often left with measuring products of the reaction of these molecules with other molecules (*e.g.*, oxidative or nitrative modification of proteins). This indirect detection of ‘footprints’ of reactive oxygen/nitrogen species makes it difficult to conclusively identify the parent species and the putative source, as well as leaving a very large black box that we are to this day only beginning to understand. The most infamous example of this black box is likely the series of clinical trials that failed to show a protective effect of vitamin E supplementation against cardiac events although all experimental data up to that point suggested that tocopherol should be protective [28]. These studies illustrate my point that there are some concepts that I believe are of utmost importance when translating oxidative stress into in vivo research.

5.1. Kinetics is king … but concentration is queen

When a compound (or protein) is called an ‘antioxidant,’ it is most often assumed that this compound directly intercepts a prooxidant (i.e., “free radical scavenger”). There are several compounds that may react very rapidly with an intrinsic ROS/RNS, and these therefore may make attractive free radical scavengers. However, to determine the biologic relevance of such reaction, it is important to also consider the achievable concentrations of the antioxidant in vivo. For homogeneous systems, multiplication of the concentration of a given antioxidant with the corresponding second-order rate constant for the reaction with the target ROS/ RNS yields the rate of disappearance of that target ROS/RNS. Such calculations yield important information on the biologic relevance of a reaction. For example, the rate constant of the reaction of $ONOO^-$ with carbon dioxide (CO_2) is on the order of $10^4\ M^{-1}\ s^{-1}$ [29]; this reasonably rapid reaction coupled with the high (~1 mM) concentration of CO_2 in biologic systems makes this reaction one of the most important reactions for determining decay of $ONOO^-$ ($10^4\ M^{-1}s^{-1} \times 10^{-3}\ M = 10\ s^{-1}$) [30]. It is a reasonable assumption that

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the maximal attainable concentration of most exogenous free radical scavengers are in the low μM range (e.g., 10 μM). Given this assumption, to significantly outcompete CO_2 for $ONOO^-$, the free radical scavenger must have a reactions rate constant $>10^6$ M^{-1} s^{-1} (10^6 $M^{-1}s^{-1} \times 10^{-5}$ M = 10 s^{-1}). Few compounds identified thus far have that rapid of a reaction.

This first-approximation approach to predict relevance of a ROS/ RNS interception reaction is informative, but doesn't take into account other aspects. First, is the product of the reaction of the scavenger with the parent ROS/RNS truly a detoxication pathway? For example, although CO_2 certainly accelerates the decay of $ONOO^-$ in vivo, ~30% of the reaction of $ONOO^-$ with CO_2 yields CO_3^- and NO_2 [29], which are more reactive ROS/RNS than $ONOO^-$ proper [31]. Second, is the reaction catalytically recyclable, or is the antioxidant irreversibly destroyed by its interaction with the ROS/ RNS? If the latter, then the antioxidant would have to be delivered or ingested so frequently that its use as a supplement may be untenable. This makes the list of exogenous antioxidants that effectively work by direct interception of ROS/RNS even shorter. This is not to say that there are not very effective antioxidants that work in biologic systems, it is simply questioning the dogma that these antioxidants mediate their protective effect by direct interception, per se.

5.2. Thinking beyond interception

It is critical to broaden the definition of an antioxidant to beyond simply those compounds that intercept free radicals. Specifically, any activity that protects against oxidative stress is by definition antioxidant, even if it doesn't directly intercept ROS/RNS. Helmut eloquently discussed this concept of defense against oxidative stress as prevention, *interception* (see above) and *repair* [32]. *Prevention* is defined as activities that prevent the shift in the balance towards oxidative stress. By this extension of the definition, activities such as administering an anti-inflammatory drug to prevent activation of the innate immune response, having ones nuclear DNA in the center of the cell where O_2 tension is the lowest, even putting on a hat to protect oneself from UVA, can all be defined as antioxidant activities. *Repair* is any activity that repairs, recycles or restitutes damage caused by oxidative stress. Both activities are arguably more kinetically favorable (and therefore more druggable) than directly intercepting free radicals.

There are compounds that may indeed improve ROS/RNS *interception* in biologic systems without directly intercepting free radicals. This can simply via maintaining catalytic antioxidant defenses, which translates to maintaining the efficient transfer of electrons from biologic sources (e.g., NADPH) to the rereduction of oxidized antioxidants [33]. For example, if one wants to study oxidative stress in the context of hepatic ischemia-reperfusion injury, it is generally necessary to fast the animals prior to the procedure [34]; else, the hepatic glycogen reserves (and therefore electron transfer to rereduce oxidized antioxidant systems) protects the organ from any significant damage. Cellular energy reserve depletion is likely a major reason that insulin resistance is associated with enhanced oxidative stress in vivo [35], and why insulin sensitizing drugs (e.g., metformin) have antioxidant effects [36]. Likewise extrinsic antioxidants may induce intrinsic antioxidant defenses; the most obvious is the induction of antioxidant genes via activation of the Nrf2/keap1 system. Interestingly,

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as most classical “free radical scavengers” are nucleophiles, they are often very good activators of the Nrf2/keap1 system and may mediate their effects via this pathway [37].

5.3. How does oxidative stress mediate damage in vivo?

The most obvious mechanism by which oxidative stress is proposed to cause injury is via chemical modification of biologic molecules. These chemical modifications can alter and/or interfere with normal biologic processes and be directly toxic to the cell. However, prooxidants can also confer cellular injury at concentrations well below observable chemical damage. For example, the amount of oxidants produced during liver ischemia/reperfusion are too low to likely cause significant direct chemical damage [38], although oxidative stress is clearly a key mechanism of injury. It is now clear that ROS/RNS can mediate and/or amplify their effects by modifying signaling cascades within the cell. As reviewed elsewhere in this issue, many signal transduction cascades are activated by oxidative stress and serve to amplify the impact of that stress. Further, the hypothesis that redox regulation of thiols is an important post-translational modification within the cell is receiving wider acceptance [39].

Lastly, some prooxidants have been identified as signaling molecules in their own right–$NO^{\bullet}$ being the most obvious example. However, many prooxidants may fall into the category of signaling molecules. Specifically, many prooxidant are regulated by enzymes, channels or pumps, are enzymatically degraded, are in rapid flux within the cell, and are specific in action, qualities which are often used to describe signaling molecules (see [40] for discussion). In summary, the cell may utilize the reactivity of ROS/RNS to rapidly alter its own signal cascades.

5.4. Impact of Helmut on modern concepts of oxidative stress in vivo

A quick summary of PubMed with the words “oxidative stress” and “in vivo” yields well over 10,000 articles, which clearly shows this is an important area of study in science. Separating the publications by year also clearly shows that this work all follows Helmut’s definition of oxidative stress [2]. The issues discussed above I believe are considered common knowledge by those with current understanding of the oxidative stress field; however it’s easy to view things accurately in retrospect. One of the things that has impressed me about Helmut is has been a consistent thought-leader in the field. Most pointedly, he was discussing these points and pushing these thoughts in the mid-80s, long before these concepts were understood in the current level of detail (e.g., [41]).

6. Summary, conclusions and personal remarks

My time at Düsseldorf had a tremendous personal impact on me. Prior to coming to Germany to join Helmut’s group, I had not left North America. I traveled all over Europe while there and it instilled in me a joy of international travel. Furthermore, as mentioned above, my German was nonexistant prior to coming to Düsseldorf. Although I’ll never be confused with being German (by a German, at least), I have a reasonably-good mastery of the language. I met one of my best friends (Lars-Oliver Klotz) at the Institut (Figs. 3 and 5). Lastly and most importantly, I met my wife through continued connections with Helmut’s Institut (she trained there 2002–2005; see Fig. 5). We have built a life together and our

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children are perfectly comfortable in either English or German and think nothing about traveling overseas. It pleases me that they learned these lessons at a much younger age than their father.

These days, there are clear metrics by which one can measure the direct impact of a scientist on the field (# publications, H-index, i10-index, etc). However, the indirect impact of a particular scientist on a field are far less tangible. Helmut has mentored several trainees over the years, several of which are now prominent in the field of oxidative stress in their own right. From my own personal experience, I know that Helmut's support of his trainees extends well beyond the time that you physically depart the *Institut*; he has been a very helpful supporter and advisor to me several times since during my career. Furthermore, Helmut has always been a very enthusiast supporter of fellow researchers and science; his service to *Archives of Biochemistry and Biophysics* is a prime example. These contributions, albeit less tangible than publications, are arguably more important in the long-term and are the true legacy of a scientist.

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Fig. 1.

My first visit to Düsseldorf. In late summer of 1997, I visited Helmut's Institut to become more familiar with his work and his team. In my spare time, I did a lot of exploring of the city. This picture is taken on the famous Königsalle, or "Kö".

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Fig. 2.
The Institut in 1998. The Institut had a research retreat in 1998 at Schloβ Krickenbeck, just near the border with the Netherlands.

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Fig. 3.

Institut Doctoral defense, 1998. The successful defense of a PhD in Germany is cause for a celebration. Here, Lars Oliver Klotz is riding his “Doktorwagen” as a newly minted PhD.

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Fig. 4.

Grant meeting, Munich, 1999. In the early part of 1999, there was a meeting to organized a collaborative research group (Sonderforschungsbereiche or SFB) in Munich. This was a series of great talks and comradery.

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Fig. 5.

The Institut and guests in 2002. The Institut hosted a celebration for Helmut's 60th birthday in the summer of 2002. The day included seminars by several former members of the Institut (e.g., Regina Brigelius-Flohé and Enrique Cadenas are in the photo). Helmut and his wife, Nancy are in the middle and their children can be seen in the background. I (right circle) visited for the celebration and to see my friend, Lars-Oliver Klotz (middle circle). My future wife, Juliane Beier (left circle), was also present … although we didn't know each other at that time.