Critical Sulfhydryl Switches, Diet, and Cancer Prevention Workshop

Executive Summary

OPENING REMARKS

Welcome

Peter Greenwald, Director, Division of Cancer Prevention
John Milner, Nutritional Sciences Research Group, Division of Cancer Prevention

Peter Greenwald, M.D., Ph.D., Director, Division of Cancer Prevention (DCP), National Cancer Institute (NCI), National Institutes of Health (NIH), welcomed participants and explained that NCI has been working to enhance the field of basic nutritional science and its relationship to cancer prevention. He noted there was evidence from the fields of epidemiology and behavioral science that diet can have an impact on cancer risk but that the literature is full of inconsistencies. A better understanding of the molecular basis for the association between diet and cancer risk be developed will help resolve these uncertainties. The food supply is undergoing fundamental changes as modern technologies that were developed for medical research are applied to food and agriculture, and it is imperative that the changes benefit the public. It is particularly important to have scientists from different fields come together to share ideas to increase our knowledge of nutritional science.

John Milner, Ph.D., Chief, Nutritional Sciences Research Group (NSRG), DCP, NCI, NIH, welcomed participants and thanked Dr. Jeffery for her efforts in organizing the workshop. He presented information on government sponsored funding—approximately $860 million—of nutrition-related research throughout the Federal Government. The NCI is spending approximately $204 million of the total, which is a considerable commitment designed to increase our understanding of nutrition and cancer risk. This effort is focused on understanding the reasons for so much variability in response to diet among populations, and how this variability translates into variable risks. This means looking at mechanisms of action of specific nutrients and diets, and focusing on nutrigenetics and nutrigenomics as potential factors for addressing this variability. There is another critical step following nutrigenetics and nutrigenomics, that of post-translational modification of proteins, that may help with our understanding of this complex issue. This workshop's focus on thiol modification of proteins may help us to understand what changes occur that account for some of the variation in response to bioactive components.

The NCI focuses on three steps in the research/translation process: discovery, development, and delivery. He noted that nutritional sciences is to a large degree in the discovery mode and thus devoting considerable attention to possible mechanism(s) by which bioactive food components work. There need to be new analytical methods and approaches applied to the discovery of these mechanisms. There also is a need to understand molecular targets and use this information to determine what factors are important for the health of the individual. Knowledge about sites of action should allow for a personalized approach to nutrition that might best be described at "nutritional preemption."

Describing the Challenge

Elizabeth H. Jeffery, University of Illinois

Elizabeth H. Jeffery, Ph.D., Professor, University of Illinois at Urbana, and a visiting scholar at NSRG, DCP, NCI, NIH, provided the perspective that in the past decade there has been growing interest in bioactive food components and diet as significant factors in the level of risk for cancer and other diseases. Dr. Jeffery recognized that participants in the workshop come from a variety of research backgrounds. In general, there are those that think about cancer biology and how thiols affect the biology in the cancer cell, and those who think about bioactive food components that are thiols or thiol-reactive compounds. The task of the workshop will be to bring these two fields of research together to gain a better understanding of the role of thiolation of proteins in effecting changes in cancer risk through diet.

Many thiols may have effects at the genomic and epigenomic levels. However, this workshop will focus on the impact of post-translational modification of proteins and the role of thiols in affecting cellular signaling through thiol redox and thiolation, just as we know that protein phosphorylation produces post-translation changes in protein activities. It is important to know how this occurs with thiols. In the diet, there are thiol-containing nutrients and non-nutrients (e.g., cysteine and allyl sulfide) that may be interacting with the thiol system. Perturbation of protein thiolation could also be the mechanism for a large number of bioactive components that do not contain thiols but are thiol-reactive, such as sulforaphane from broccoli or selenium-containing compounds.

Dr. Jeffery reviewed the agenda and listed the subjects to be covered in each session of the workshop:

Session 1: Setting the Scene: Differentiating between thiol-active redox reactions and reactive oxygen species (ROS)/ oxidative damage;

Session 2: Technologies that can be applied to this area;

Session 3: Diet and thiol regulation;

Session 4: Molecular mechanisms that may be involved in thiol regulation;

SESSION I: SETTING THE SCENE

New Frontiers for Antioxidants
Douglas E. Brash, Yale School of Medicine
Cysteine/Glutathione Deficiency Disease
Leonore A. Herzenberg, Stanford University

Douglas E. Brash, Ph.D., Professor, Department of Therapeutic Radiology and Genetics, Yale School of Medicine, New Haven, Connecticut, presented an overview of antioxidants and discussed ways in which the study of sulfhydryl switches differs from the classic understanding of oxidant and antioxidant biochemistry, suggesting new frontiers in protein regulation. Leonore A. Herzenberg, Ph.D., Professor, Department of Genetics, Stanford University, California, presented information on pathologies associated with thiol deficiency, and the health benefits associated with thiol-containing medications such as N-acetyl cysteine (NAC).

A critical issue in the study of sulfhydryl switches is to understand the interplay between oxidants/antioxidants and the thiol redox system within the cell. For example, can vitamins E and C alter sulfhydryl switches? Addition of many different exogenous compounds (oxidants, antioxidants, reactive metal ions or reactive toxicants such as the quinone imine metabolite of acetaminophen) can readily disrupt normal thiol redox regulation within the cell. This can be either by altering redox or by depleting redox components such as cysteine or glutathione molecules. On the other hand, natural redox modulation within a cell, undistorted by exogenous compounds, may be more subtle, responding to changes that do not reflect the status of the entire cell, and that are rapidly returned to normal: sulfhydryl switches that are switched on, initiate a cascade of events, and are then switched off again. For example, many transcription factors are sensitive to change in redox, such as p53 with its 10 cysteine molecules in the DNA binding domain, open to modification by endogenous mechanisms such as interaction with the thioredoxin reductase- Ref-1 pathway. The importance of thiol status in these signaling molecules is highlighted by the fact that they each require selenium, the biochemistry of which is intimately involved in thiol interactions. Another example of the importance of thiol switches in regulation of intermediary metabolism is the oxidation of protein sulfhydryls which appears to be a common signal for protein degradation.

To date, it would appear that all too often the study of the role of sulfhydryl switches in signal transduction has been confounded by gross redox changes triggered by addition of exogenous oxidant tools. Such effects can, of course, be prevented by an excess of antioxidants such as vitamin E either exterior or interior to the cell, but such studies are unlikely to reflect any physiological role of vitamin E. Such massive changes in redox can alter the fine balance of proteins normally regulated by thiol redox such as p53, which in response to small redox changes can switch between DNA repair/ proliferation and apoptosis. Subtle redox changes that more typically regulate cellular pathways, rather than acting directly, may be part of a strictly regulated cascade of metabolic changes.

Given then, that thiol regulation is distinct from the action of pro-and anti-oxidants and that its study may be confounded by oxidative damage that can disrupt the thiol balance, disease models rather than chemical intervention models, may be most useful in the study of the biochemistry and physiology of sulfhydryl switches. Cellular metabolism includes an intricate redox regulatory system based on thiol switches that is altered in multiple diseased states and may at times be reversed by feeding a thiol-rich diet or providing medication such as NAC. Oral NAC has been studied in over 50 clinical

trials and has, for example, been found effective in improving the immune response in AIDS patients, as well as in a number of other disease states such as cystic fibrosis, for which NAC was first developed. Whether physiological improvement is dependent upon the ability of the drug or diet to alter cellular redox directly, or to provide the essential components such as cysteine, remains to be determined. One possible route for investigating this is to study protein thiolation in different disease states and in the reversal of disease during therapy. The study of thiol physiology and biochemistry has not previously been considered as a whole, possibly because it cuts across multiple fields, including genomics, proteomics, and metabolomics. A listing of major thiol targets would likely be useful in establishing the field.

Another concern raised in this session was the need to understand personal differences in thiol status. Epidemiological studies show that dietary antioxidants can decrease cancer risk only to about 0.6 in the general population, whereas individuals at high risk (elderly, smokers, and possibly antioxidant-deficient individuals) can see a drop in risk to about 0.3 of their original risk. Dr. Brash suggested that this may reflect a redundancy in the metabolic pathways in the general population, so that if one pathway fails, another is able to maintain the status quo. If one of the pathways is absent in individuals described as at high risk for a disease, then a disruption of the remaining pathway, even if it is only temporary, can be devastating – whereas continual enhancement of the remaining pathway can relieve the risk caused by the failing alternative pathway. This concept is supported by studies comparing single and multiple knock-out models of cancer. Should some component associated with thiol redox (e.g., glutathione, cysteine, selenium) become limiting, this could jeopardize the proper functioning of a pathway – and if the system has previously lost alternatives due to genotype or environmental factors, this could prove fatal. Alternatively, providing excess of these factors in the diet could overcome the need for the second, non-functional pathway, allowing lowering of risk. Personalized nutrition, based on alleviating faulty pathways, will have an important future in maintaining health in individuals at high risk for certain diseases.

SESSION 2: EMERGING TECHNOLOGY FOR THE STUDY OF CELLULAR SULFHYDRYLS

What Methods Are Available for Clinical Evaluation of Thiol Status?

Martha H. Stipanuk, Cornell University

How Can We Evaluate Redox in Whole Cells: State of the Art and Novel Approaches

Martin A. Philbert, University of Michigan

Martha H. Stipanuk, Ph.D., Professor, Division of Nutritional Sciences, Cornell University, Ithaca, New York, presented information on the methods available today for clinical evaluation of thiol status, capabilities and deficiencies. Martin A. Philbert, Ph.D., Associate Professor of Toxicology and Associate Chair, Department of Environment Health Sciences, University of Michigan, Ann Arbor, presented information on novel approaches to evaluate redox in whole cells, focusing on nanotechnologies under development.

The study of sulfhydryl switches demands that we have good tools for the estimation of redox-sensitive components. Blood is the most readily available tissue for clinical study of thiol status. There are several key technical concerns in the measurement of redox molecules, as well as concerns over interpretation of the relevance of plasma readings to report on intracellular thiol biochemistry. Key to accurate estimation of the oxidation status of redox couples is the use of iodoacetate (slow), monobromobimane or N-ethylmaleimide to bind free SH groups, thus arresting oxidation and inhibiting re-equilibration. There may be additional plasma components that interfere with the sulfhydryl status of a sample, including hemolysis (erythrocyte glutathione levels are far greater than plasma levels), γ-glutamyl transpeptidase and free metals. A useful technique is to derivatize the amino group rather than the sulfhydryl group, since this allows concomitant measurement of the reduced and oxidized couples of cysteine and glutathione.

Cysteine (250 μmol/L) constitutes greater than 80% of plasma thiols, whereas glutathione and thioredoxin become more important intracellular players, in the cytosol and nucleus, respectively. During this session, a picture of the relationship between the extracellular and intracellular environments started to emerge: extracellularly, the cys/ cySS (cytsteine/ cystine) couple is predominant, and typically more oxidized than the GSH/ GSSG couple. Intracelluarly, the now predominant GSH/GSSG couple is always more reduced than in the extracellular compartment. Yet even given these many differences between plasma and intracellular thiol status, measurement of plasma redox couples is not without merit, since changes in plasma redox are reported with altered physiological states. For example, chemotherapy is associated with oxidation of the plasma GSH/GSSG couple, and smokers have been reported to have a significantly greater oxidized plasma cys/cySS couple than either non-smokers or former smokers. Even aging alone is seen to cause a shift in the plasma cys/cySS ratio to a more oxidized state, although interestingly this becomes evident at a younger age than any shift in the plasma GSH/GSSG ratio, which is not evident until after about 45 years of age.

In considering measurement of tissue glutathione, one runs into the problem that different cell types, or even similar cell types at different physiological stages, may have very different glutathione levels or redox states, requiring separate measurement. One way to overcome this is to study a monoculture. However, this also is confounded by the fact that typically cell culture is carried out under air (21% oxygen). Whereas this is not

too different than the environment of a lung cell (~16%), it differs considerably from the 5 – 9% oxygen more common for other tissues within the body. Culture under hypoxic (or whole-body normoxic) conditions is possible, yet still there is a concern that the redox status is altered as soon as one disrupts the normal physiology of the cell. One way to overcome this may be to turn to nanotechnology, placing redox sensors within the living cell.

Fluorescent dyes have been used as indicators of various metabolites (e.g., calcium) within living cells. However, the use of fluorescent dyes for evaluation of redox is problematic, since frequently they bind to the cellular components under study, altering the environment that they were intended to evaluate. Furthermore, the sensitivity of fluorescent dyes to reflect thiol status within hydrophobic and hydrophilic environments within the cell may be very different, providing non-interpretable data when mixtures are viewed as one dataset. Other parameters that may similarly cause these dyes to provide unequal responses are subcellular changes in pH, ion concentration, membrane potential, or simply lack of diffusion allowing compartmentalization. Dr. Philbert and colleagues are developing a series of micro-probes termed Probes Encapsulated by Biologically-Localized Embedding (PEBBLEs). These sensors appear to be sufficiently sensitive to be used at concentrations that do not perturb the natural redox by their presence, and the matrix can be altered to meet the needs of the study environment (i.e., hydrophobicity etc.). PEBBLEs are also under development to act as biosensors for a number of other cellular endpoints, unrelated to redox. A series of sensors that signal at different redox potentials could, in real time, show how various compartments of the cell fluctuate back and forth through redox changes, or show how tumor and normal cells vary during passage through the cell cycle.

SESSION 3: DIET AND THIOL REGULATION OF THE CELL CYCLE

Do Cancer Cells Have an Aberrant Thiol Status In Vivo That Responds to Diet?
Garry R. Buettner, University of Iowa
Does Dietary Cysteine Regulation of the Cell Cycle Provide Clues to Additional Dietary Influences?
Dean P. Jones, Emory University
How Do Dietary Alterations in Glutathione and Thioredoxin Levels Affect Cell Cycle?
Arne Holmgren, Karolinska Institute

Garry R. Buettner, Ph.D., Professor, Department of Radiation Oncology/Free Radical and Radiation Biology, University of Iowa, Iowa City, described the changes in thiol status as a cell progresses from a proliferating stem cell, through differentiation to end in apoptosis, and the finding that cancer cells differ from non-transformed cells in their redox status. Dean P. Jones, Ph.D., Professor, Department of Biochemistry, Emory University, Atlanta, Georgia also discussed redox regulation of the cell cycle, focusing on changes effected by dietary cysteine. Arne Holmgren, M.D., Professor, Medical Biochemistry and Biophysics/Medical Nobel Institute for Biochemistry, Karolinska Institute, Stockholm, Sweden, presented further information on cancer cell proliferation and cell cycle changes related to diet-directed alterations in intracellular GSH and thioredoxin.

Studies on diet, antioxidants, and redox status reflect a relationship between sulfhydryl biochemistry and cancer, although they rarely address the topic directly. Results of these studies indicate that dietary antioxidant factors, including lycopene and other carotenoids and flavonoids have been implicated in mitigating the progression of prostate cancer. Whereas many "antioxidant anticarcinogens" like these are possibly best recognized for their effects on the induction of Phase II detoxification enzymes, they also cause elevation of tissue GSH levels, demonstrating the potential for an effect on sulfhydryl switches, as a mechanism for their influence on cancer risk.

Normal cells are metabolically highly reduced during proliferation. As these cells progress from proliferation to differentiation, they pass to a more oxidized state. As differentiation is completed, these cells return to a more reduced state. However, cells that progress further to apoptosis do not undergo reduction, but become further oxidized. Research is needed to determine whether changes in redox are instrumental in directing the cell through these various stages. Experiments suggest that the redox of the proliferating cancer cell is shifted to a more oxidized state, one that in normal cells would be associated with the initiation of differentiation. That this switch to differentiation does not occur in the cancer cell is clear – but whether further manipulation of the redox status can trigger such a switch, or whether the switch(es) that normally respond to an oxidative change in a non-cancerous cell are missing in the cancer cell is unclear. Several classic dietary antioxidants, such as selenium and lipoic acid are known to increase antioxidant enzymes like glutathione peroxidase, but whether this is implicated in the anticarcinogenic actions of these antioxidants is unknown. Models are needed that allow the study of redox in an environment more closely reflecting oxygen tensions found in tissues. Such models can be used to define the pathways triggered by redox changes, providing an understanding of whether changes in redox occur as a result of passage through the cell cycle, or if redox, at least in part, directs passage through the cell cycle.

Looking in more depth at the changes from reduced to oxidized as the cell passes from proliferation to differentiation and apoptosis, one sees that whereas this is true for

glutathione, that the cys/cySS couple remains oxidized through out the cell cycle, and that thioredoxin, in nucleus but also in cytosol and the mitochondrion, tends to stay more reduced throughout the cell cycle. Thus not only may redox be compartmentalized within the cell, but depending upon the precise protein environment, differential oxidation of protein sulfhydryls would be expected to occur. Regulatory mechanisms could allow a series of proteins to be activated and then inactivated as the cell progressed from more reduced to more oxidized. For example, even under cellular conditions associated with apoptosis, thioredoxin remains reduced. Whether this is due to the protein environment or to subcellular compartmentalization of factors causing a redox change is unclear. What is clear is that the glutathione/glutaredoxin pathway for reduction of cellular components is quite separate from the thioredoxin pathway of reduction. Thus in a single cell at a given intracellular redox, a sulfhydryl switch could be reduced by thioredoxin and then oxidized by glutathione. Furthermore, cellular proliferation may be regulated by both intracellular and extracellular thiol status: It is known that thiol status regulates expression of both Fas death receptor and the Fas ligand, on the exterior surface of the cell membrane.

Without protein cofactors, a mix of glutathione and cysteine couples will eventually reach redox equilibrium; although this is a slow process. This helps to understand that in the cell, one can have a relatively oxidized cysteine couple, a relatively reduced thioredoxin couple, and that the greatest range for variation and therefore for physiological effects is associated with glutathione. Entrance of cysteine into the cell might be sufficient to allow oxidation without the need for ROS initiation, so that by regulating cysteine influx, the cell might regulate passage from proliferation to differentiation. In studies designed to do just this, one finds that growth factors are completely able to over-ride any cysteine redox effects, reminiscent of the redundancy theory of alternative pathways.

SESSION 4: SITE-SPECIFIC MODIFICATIONS OF CELL SIGNALING PROTEINS BY SULFHYDRYL SWITCHES

Can Diet Modify Cell Proliferation Through Sulfhydryl Switches on Transcription Factors?

David Gius, National Institutes of Health

Can Dietary Factors Regulate Activity of Cell Signaling Proteins Through Sulfhydryl Biochemistry?

Catherine A. O'Brian, University of Texas, M.D. Anderson CC

How Can Non-Thiol Dietary Components Cause Thiol Regulation of the Cell Cycle?

Chung S. Yang, Rutgers University

David Gius, M.D., Ph.D., Section Chief, Molecular Radiation Oncology, Radiation Oncology Branch, NCI, NIH, took us inside the nucleus, to consider the role that redox regulation plays in regulating immediate early genes and transcription factors that are constitutively upregulated in tumor cells because they up-regulate proliferation and pro-survival. Catherine A. O'Brian, Ph.D., Professor, Department of Cancer Biology, University of Texas M.D. Anderson Cancer Center, Houston, discussed enzymes belonging to the protein kinase C family that are regulated by glutathiolation, and the potential for influence of diet on these sulfhydryl switches. Chung S. Yang, Ph.D., Professor and Chair, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers University, Piscataway, New Jersey, broadened our perspective by discussing non-thiol dietary components that may perturb thiol regulation of the cell cycle.

In response to oxidative stress, a series of immediate early response genes, including the oncogenes *c-fos* and *c-jun* are upregulated, resulting in enhanced expression of the pro-proliferation transcription factor AP-1, a heterodimer of Fos and Jun. Both AP-1 and the signaling factor redox factor-1 Ref-1 contain redox-sensitive cysteines that may regulate their activities in response to oxidative stress: specifically, it appears that the binding of AP-1 to DNA is redox-sensitive. Similarly, both thioredoxin reductase and thioredoxin bear cysteine-rich redox-sensitive domains. Transfection and overexpression of thioredoxin reductase enhances AP-1 binding, whereas inhibition of thioredoxin reductase interrupts this activation. Furthermore, overexpression of a mutant thioredoxin reductase that was missing these redox-sensitive cysteines, was without effect. Thioredoxin reductase may therefore be intimately involved in the response of these factors to oxidative stress, possibly through reduction of thioredoxin, which may then translocate into the nucleus to combine with Ref-1 in supporting AP-1 binding. These redox-sensitive signaling proteins may play an integral part in the balance between pro-oxidant and anti-oxidant systems, directing the cell toward survival/DNA repair or apoptosis, following oxidative damage. The mechanism whereby oxidative stress triggers reduction of thioredoxin reductase and Ref-1, remains speculative, but may involve an enhancement of NADPH generation.

Members of the protein kinase C (PKC) family mediate tumor promotion and progression. They contain between 16 and 28 cysteine residues, and are regulated by glutathiolation. Redox regulation of the different isoforms of PKC shows that, at least in this system, glutathiolyation can inactivate one protein, whereas an opposing isoenzyme was resistant to loss of activity due to glutathiolation. When cells were placed under oxidative stress by including diamide in the medium, glutathiolation of PKC occurs. Specifically, PKCδ, an enzymes that normally inhibits promotion, did not lose activity

when glutathioloated, whereas PKCε, an enzyme whose activity is to enhance promotion, was inhibited by glutathiolation. Thus glutathiolation inhibits PKC-dependent promotion. This action of glutathione to switch off promotion has been modeled *ex vivo*, in cell culture, as well as in whole mice, using diamide, cystamine or cystine, and NAC was able to reverse this. It will be interesting to see if dietary thiol-containing and thiol-reactive compounds that are associated with prevention of carcinogenesis, such as allyl sulfides and isothiocyanates, can also trigger these changes in PKC activity in mice.

Tea polyphenols, including the major component epigallo catechin gallate (EGCG) have both antioxidant and anticarcinogenic activity. However, as with so many dietary antioxidants with possible anticarcinogenic activity, whether there is a relationship between antioxidant and anticarcinogenic activity is not known at this time. There are a number of theories based on quenching of reactive oxygen species, but these have not been proven and, at least in cell culture, EGCG appears to have its pro-apoptotic effects when acting as a prooxidant. EGCG addition to cell culture causes production of peroxide, and catalase inhibits EGCG-induced apoptosis of H661 human lung cells. It is not known if EGCG acts in this pro-oxidant fashion *in vivo*.

SUMMARY

1. Thiol-reactive compounds, presently recognized as antioxidants appear to have a role in cellular physiology, protecting against carcinogenesis. Information is incomplete, but points toward thiol-rich foods being able to provide such protection.
2. Quenching or reversing the action of radicals, such as reactive oxygen species, is not the full story behind the health benefits of the bioactive food components loosely described as "antioxidants."
3. Sulfhydryl oxidation/reduction and/or thiolation causing redox modifications of cellular proteins regulate a wide number of cell pathways associated with proliferation, differentiation and apoptosis.
4. There is not a constant, or even a single, redox equilibrium within cells-and redox changes with cell type and cellular health status, such as damaged or aging cells. It brings in activity of a cys/cySS couple both inside and outside the cell, as well as glutathione and thioredoxin couples.
5. Cell culture is carried out at falsely high oxygen tensions that may confound the study of regulation by redox. Methods for studying thiols and thiolation need further development.
6. Dysfunction of redox systems such as GSH/GSSG is an integral part of the manifestation of many diseases: redox changes due to disease state and/or aging could be an early biomarker used as a measure of health and wellness.
7. Redox status can be altered by certain radicals and other chemicals, including sulfur amino acids and a number of bioactive food components thought of as antioxidants.