CHAPTER 13

MULTIDISCIPLINARY ASPECTS OF REGULATORY SYSTEMS RELEVANT TO MULTIPLE STRESSORS: AGING, XENOBIOTICS AND RADIATION

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Abstract: Free-radical biology, which is central to the fields of radiation, aging and xenobiotics, has shifted from a paradigm highlighting damage to a paradigm emphasizing the role of free radicals in regulatory processes. A unified approach is possible since multiple stressors tend to activate a coordinated set of common mechanisms. These include antioxidant defenses, metal chelators, DNA repair systems, heat-shock proteins, xenobiotic efflux transporters, protein degradation systems, cell survival and apoptosis pathways and detoxification systems. Nearly all MAPK signal transduction pathways employ oxidative signaling, largely generated via membrane-bound NADPH oxidase systems. These regulate most cellular stress, growth and apoptotic responses. A new global perspective highlighting "Electroplasmic Cycles" incorporates numerous cellular aspects of control including free radicals, protein and histone modifications, nuclear–cytoplasmic transport, and ion channels. Aspects critical to multiple stressors include complex interactions related to apoptosis-necrosis, immunological responses (Toll-like receptors), bystander effects, chaperone proteins, and multiple xenobiotic efflux proteins. The synthesis suggests that a systems approach to multiple stressor impacts is required since understanding requires holistic appreciation of integrated regulatory circuitry.

Keywords: radiation; xenobiotics; aging; free radicals; signal transduction; redox; ion channels; growth; chaperone proteins; multiple drug resistance; apoptosis; clearance; Toll-like receptors; auto-immunity; nuclear–cytoplasmic transport

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The Regulatory Paradigm

Free-radical and radiation biology are rapidly expanding from a paradigm emphasizing damage, protection and repair (see McBride et al., 2004) to encompass the pervasive regulatory impacts of reactive oxygen, nitrogen, and peroxidation species (e.g. Fornace, 1992; Herrlich et al., 1999; Schmidt-Ullrich et al., 2000; Bauer, 2002; Droge, 2002, 2005; Dent et al., 2003; Finkel, 2003; Esposito et al., 2004; Balaban et al., 2005; Cai, 2005; Fedoroff, 2006; Meng et al., 2006). This is globally applicable to growth, development, organismal and cellular function, aging, stress, xenobiotics, radiation, immunology, transformation and numerous pathologies. Regulatory impacts of radiation and xenobiotics trace largely to generation of free radicals from water by radiation and xenobiotic detoxification (e.g. p450 monooxygenases). Metals are particularly important pollutants as they synergize conversion of relatively benign reactive oxygen species such as superoxide and hydrogen peroxide to the more harmful hydroxyl radical (e.g. iron and Fenton chemistry). Very small changes in transition metals can have large impacts such as alterations in p53 function (Meplan et al., 2000). Moreover, antioxidants, particularly the glutathione (GSH) system, importantly regulate cellular redox status. All of these aspects are also incorporated in the prevailing free-radical theory of aging which predicts that chronic production of endogenous free radicals from mitochondria, NADPH oxidases (NOX) and other processes results in damage to DNA, proteins and lipids. Accumulating damage impacts cellular constituents, organelles, extracellular matrix, tissues and organ systems resulting in an aging organismal phenotype.

Radiation, xenobiotics and aging are unified by their joint linkage to free-radical biology. In fact, the free-radical theory of aging was derived directly from radiation biology (Harman, 1956). Diverse stressors (e.g. free radicals, radiation, heat, toxins, ionic disruption, dietary restriction, and even severe sleep deprivation) invoke a generalized and coordinated upregulation of numerous defensive systems (Jazwinski, 1996; Martin et al., 1996; Rollo, 2002; Gems and McElwee, 2005). These include antioxidants, metal chelators, heat-shock proteins, DNA repair systems, protein degradation systems, P450 monooxygenases, glutathione-S-transferases, apoptotic pathways and multiple drug resistance (efflux) proteins. Most models of extended longevity express stress-resistant phenotypes, and selection for multiple-stress resistance can yield extended maximal longevities (Wang et al., 2004; Frankel and Rogina, 2006; Stuart and Brown, 2006; Wang and Tissenbaum, 2006).

Cysteine–Methionine Residues, NADPH Oxidase and Redox Regulation

Advances in free-radical-mediated signaling have involved parallel increases in understanding NOX and redox-sensitive residues of diverse regulatory peptides (Scmidt-Ullrich et al., 2000; Bauer, 2002; Droge, 2002, 2005; Dent et al., 2003; Cai, 2005; Kuroda et al., 2005). Mitochondria are generally considered the main source of cellular free radicals (see Balaban et al., 2005) but NOX isoforms are highlighted more in signal transduction. Regardless, mitochondrial function and NOX are linked via mutual impingement on NAD–NADH and the associated cellular redox environment (Rollo, 2006). Redox state regulates numerous signal transduction and transcription factors largely via reversible oxidation or reduction of sensitive cysteine and methionine residues. General oxidative inhibition of tyrosine phosphatases means that cell signal transduction via antagonistic phosphatases and kinases is redox dependent. Tyrosine phosphatases can also recognize phosphorylated serine/threonine residues (Meng et al., 2006).

Redox balance and associated cysteine residue status are mainly modulated by the glutathione antioxidant system. Thioredoxin and glutaredoxin augment GSH reduction of critical residues in signaling molecules (NOX-dependent processes) that control ion channels, the cell cycle, apoptosis, mitochondrial functioning, cytoplasmic-nuclear shuttling (e.g. RNA and transcription factors), stress, repair and degradation systems, secretion, excretion and nutrient uptake. Coordinated temporal cycling of numerous important functions is closely linked to shifts between oxidative and reducing redox status (with the reduced state being basal). This includes cycles involving membrane polarization, intracellular pH, calcium and potassium levels. Complications include localized alterations in redox status within various cellular compartments. Oxidative stress from multiple stressors is likely to trigger multiple mechanisms and pathways simultaneously (see Dent et al., 2003). Although some aspects may allow protective compensation, general upregulation of redox-sensitive systems is likely to be disruptive.

The co-repressor, carboxyl-terminal binding protein (CtBP) regulates numerous aspects of development, the cell cycle and transformation via binding to transcriptional repressors. Such binding is positively linked to NADH, allowing CtBT to serve as a redox sensor (Zhang et al., 2002). Furthermore, some NOX isoforms are localized to the nucleus where they generate SOR. In endothelial cells nuclear NOX4 activity increases the transcription of genes containing the MARE response element that coordinates part of the oxidative stress response (Kuroda et al., 2005). Since cellular redox state and NOX function both hinge on NADH, Kuroda et al. (2005) suggest that NOX4 also functions as a nuclear redox sensor. Silencing of NOX4 prevents

induction of the "unfolded protein" stress response (Fedoroff, 2006). NOX4 may also contribute to the cellular senescence phenotype via alterations which may include impacts on telomeres.

SOME FEATURES REGULATED BY REVERSIBLE OXIDATION/ NITROSYLATION OF PROTEIN CYSTEINE AND METHIONINE RESIDUES

Pathways: (MAPK-ERK, PI3K, JAK-STAT, p38, JNK)
Growth factors, cytokines
Mitochondrial complex I
Creatine kinase
Caspases
Tyrosine phosphatases (e.g. PTP1B, PTN, SHPs, MKPs)
Ion channels (many)
Nuclear transport (import and export recognition sequences)
Transcription factors (e.g. AP-1, p53, NFκB, HIF-1α, glucocorticoid receptor)
Keap-Nrf2-ARE phase 2 genes
Protease inhibitors
Proteases
Glutathione, thioredoxin, peroxiredoxin
Thiol peroxidase
Chaperones

Ion Channels, Free Radicals and Radiation

Whereas the free-radical theory of aging has dominated gerontological and radiation research, in the realm of neurodegenerative disease and aging, a paradigm highlighting Ca^{2+} dysregulation has also predominated for at least 25 years. An ion channel perspective may in fact be expanded to subsume free-radical biology. Presumably ion channels were already crucially deployed in cells living in ancient reducing environments, with redox regulation being elaborated for protection against radiation (before the ozone layer) and as atmospheric oxygen increased. The basal reducing state of cells likely reflects this early heritage, which is at least as ancient as the preservation of cellular ionic conditions resembling seawater. Many pesticides impact ion channels and can alter numerous features including brain neurochemistry (particularly the dopamine system) (Richardson et al., 2006). Most antibiotics (like Rapamycin) impact ion channels. Furthermore, nearly all evolved toxins and venoms target channels, and at least one third of medicinal drugs act on channels (Pardo et al., 2005). The association of NOX with ion channels links free-radical production directly to ion channel activities, and a voluminous literature documenting the involvement of NOX isoforms

in obesity, diabetes, atherosclerosis, cancer, neurotransmission, long-term potentiation and memory, most neurodegenerative diseases, growth factor signaling, cellular pH and redox state, immunological activities and aging.

Radiation deposition of energy into cells causes some direct damage but it also lyses water to form free radicals (including damaging hydroxyl radicals). Low-level γ-irradiation activates outward K^+ channels within seconds. This is associated with generation of free radicals by radiation, and the response is inhibited by N-acetyl cysteine. K^+ channel activation is implicated in other stress responses such as to heat or hydrogen peroxide (Kuo et al., 1993). K^+ channels are regulated by reversible oxidation of sensitive cysteine and methionine residues (Ruppersberg et al., 1991; Chen et al., 2000). This can modulate neuronal excitability. Reactive oxygen species generally allow Ca^{2+} entry and disrupt Ca^{2+} homeostasis in a variety of cell types (Huang et al., 2003). ROS generally impair and antioxidants often protect ion transport proteins underlying transmembrane signal transduction. Kourie (1998) reviews such impacts for (i) ion channels such as those for Ca^{2+} (including voltage-sensitive L-type Ca^{2+} currents, dihydropyridine receptor voltage sensors, ryanodine receptor Ca^{2+}-release channels, and D-*myo*-inositol 1,4,5-trisphosphate receptor Ca^{2+}-release channels), K^+ channels (such as Ca^{2+}-activated K^+ channels, inward and outward K^+ currents, and ATP-sensitive K^+ channels), Na^+ channels, and Cl^- channels; (ii) ion pumps, such as sarcoplasmic reticulum and sarcolemmal Ca^{2+} pumps, Na^+-K^+-ATPase (Na^+ pump), and H^+-ATPase (H^+ pump); (iii) ion exchangers such as the Na^+/Ca^{2+} exchanger and Na^+/H^+ exchanger; and (iv) ion cotransporters such as K^+–Cl^-, Na^+–K^+–Cl^-, and P_i–Na^+ cotransporters.

Many of the features listed earlier for free-radical processes have very broad impacts on cell functions in their own right. Consider ion channels:

MAJOR FUNCTIONS REGULATED BY ION CHANNELS

- Cell size
- Osmotic pressure
- Electrical polarization, depolarization
- Nutrient uptake (including amino acids)
- Neuroendocrine secretion and re-uptake
- Regulation of pH
- Excretion, detoxification
- Muscle contraction
- Redox control
- Aquaporins transport (including hydrogen peroxide)

All of the earlier circumstances highlight a critical linkage between free-radical processes, ionic alterations, channel functions and electrical properties. Consequently, the temporal organization of cells might well reflect "Electroplasmic Cycles." Many critically important cell transduction-transcription factor pathways are redox-ion regulated and sensitive to the impacts of radiation (particularly ionizing radiation) and xenobiotics (see later). A global perspective reveals the existence of redox-associated cycles that dichotomously coordinate major organismal and cellular functions. Probably the most obvious resolution of such cycling is circadian (24 h) rhythmicity. This is associated with endogenous clocks and the antagonistic functioning of the waking versus sleeping states. These states in turn represent opposing watersheds of regulation of various functions by the stress hormone axis in waking versus the growth hormone axis in sleeping (The clockwork genome hypothesis – Rollo, 2007). Although the circadian rhythm is the most obvious expression of endogenous rhythmicity, it is actually composed of and emerges from shorter-duration cycles. Of these, the ultradian rhythms of foraging-feeding and sleep (period lengths of 3–4 h) are well established. Even finer resolution of cycling can be found; however, some examples of nuclear shuttling being remarkably fast. This may allow both nuclear import and export to be carried out within relatively short time frames. Rhythmicity is critical for a multiple stressor paradigm since biological systems respond differently at different cycle phases (e.g. the effectiveness of drugs and detoxification systems shows strong circadian variation).

Yin–Yang Cycles of Redox and Cell Functions (The Electroplasmic Cycle)

Oxidizing	Reducing
Elevated reactive oxygen species	Lower reactive oxygen species
Elevated reactive nitrogen species	Lower reactive nitrogen species
Increasing GSSG/GSH ratio	Lower GSSG/GSH ratio
NAD(P)H oxidase activity Reduced	NOX activity
Acidification	Alkalization
Catabolism	Anabolism
Activity, output	Inactivity, sleep (stimulated by GSSG)
Depolarization	Hyperpolarization
Elevated intracellular Ca^{2+}	Reduced intracellular Ca^{2+}
Decreased intracellular K^{+}	Increased intracellular K^{+}
Kinase activity	Phosphatase activity
Acetylation (p300/CBP)	Deacetylation (HDAC, SIRTs)
Oxidized cysteine residues	Reduced cysteine residues
Oxidized methionine residues	Reduced methionine residues
Nuclear import (e.g. AP-1, NF-κB)	Nuclear export (AP-1, NF-κB)

Spatial Compartments

Besides temporal aspects, organ, tissue and intracellular compartmentalization is crucial to specialized functioning. Spatial compartmentalization of glutathione, particularly cytosolic versus mitochondrial pools, may be highly relevant to radiation. Lack of clear dose-response relationships for low dose radiation is an outstanding quandary for radiation biology. One factor may be the presence of a large effective buffer of reduced glutathione such that discernable impacts require 40–50% depletion of mitochondrial GSH (Han et al., 2003). Initial depletion of mitochondrial GSH has little effect but a dose-response relationship in mitochondrial H_2O_2 production emerges beyond a threshold of 50% GSH depletion. A further compensation to redox stress involves the anti-apoptotic function of bcl-2. Besides raising cellular levels of GSH, bcl-2 redistributes GSH from the cytosol into the nucleus. Whereas the nucleus normally sequesters ~30% of cellular GSH, bcl-2 upregulation increases this to ~75% (Voehringer et al., 1998). This mechanism would probably antagonize radiation impacts, particularly at low doses, and like GSH buffering in mitochondria, yield a threshold response to increasing radiation dosage. Ubiquitination is also partially regulated by spatial localization. The ubiquitin-conjugating enzyme is transported into the nucleus if charged with ubiquitin (Sommer and Jarosch, 2005). Regardless of the impacts of cytosolic redox status, the DNA binding of many transcription factors (e.g. AP-1, p53, NF-κB) require reduction of sensitive cysteine residues in the DNA binding region. The co-activator, Ref-1 serves to reduce such cysteines and synergizes transcription (Meplan et al., 2000). Such activity could act in concert with the import of GSH by bcl-2, creating some dichotomy between cytosolic and nuclear regulation.

Cytoplasmic–Nuclear Transport

Of critical importance to both spatial and temporal regulatory impacts is cytoplasmic–nuclear transport of materials into or out of the nucleus (Macara, 2001). This applies to histone protein H1, actin, ribosomal proteins, Cyclin B1, p53, Smad, PKA, PKC, FOXO, MEK1, ERK, c-fos, protein kinase inhibitor α, p38, STAT1, helicase, STAT5, androgen receptor, parathyroid hormone-related protein, fibroblast growth factor, p53, human telomerase, NF-AT4, histone deacetylase2/6, tRNA, and mRNA (Kudo et al., 1999a,b; Verdel et al., 2000; Strom and Weis, 2001; Poon and Jans, 2005; Wiedlocha et al., 2005). Many of these elements shuttle between the cytoplasm and nucleus, highlighting

the existence of (electroplasmic) regulatory cycles that appear to be correlated to redox. Among elements that are subject to shuttling via importing family peptides are molecular components of the circadian clock such as mammalian Cryptochrome 2 and mammalian Period-2. Transport processes are an essential aspect of the transcriptional oscillations and auto-regulatory feedback by translated proteins that derive clock function (Sakakida et al., 2005). Clocks in turn diversely regulate cellular and organismal functions, and disruption can lead to cardiovascular disease, psychiatric disorders and cancer (Sakakida et al., 2005).

Cytoplasmic retention of particular proteins can trace to heat-shock proteins like HSP90 (Jans, 1995; Poon and Jans, 2005). Transport proteins belong to the large importin-β-family (at least 22 members) (Strom and Weis, 2001). The GTPase, Ran, binds importin in the nucleus, inducing release of cargo. Gradients of Ran may also regulate directionality of transport. Importin-β proteins contain sequences for recognizing Ran, cargo and nucleoporins (Kudo et al., 1999a; Strom and Weis, 2001; Petosa et al., 2004; Fahrenkrog, 2006). The nuclear pore complexes may be the largest protein structures in eukaryotic cells (Macara, 2001).

Importin-β-family proteins contain sensitive cysteine residues that are crucial for functioning. Various transport recognition sequences may also have such cysteine residues, and yet consideration of regulation by reversible oxidation or glutathionation appears relatively unexplored. Regardless, there are several suggestive examples of reversible oxidation of cysteine residues as key modulators of nuclear–cytoplasmic transport. Pap-1, a fission yeast homologue of Jun is maintained in the nucleus by oxidative stress, and this traces to oxidation of critical cysteine residues. Pap-1 is involved in free-radical responses, metal detoxification and multiple drug resistance acting via catalase, thioredoxin, thioredoxin reductase, glutathione reductase and ABC transporters (Multiple drug resistance proteins) (Kudo et al., 1999b). The yeast multiple drug transporters are homologues of human p-glycoprotein encoded by the MDR1 gene that is associated with JNK activity (Toone et al., 1998). Yap1p is also homologous to mammalian AP-1 (Jun) in budding yeast and regulates responses to oxidative stress and cadmium toxicity. The nuclear export signal of Yap1p is regulated by redox modulation of reversible disulfide bond formation in critical cysteine residues. Kuge et al. (2001) suggest that nuclear localization may be regulated by thioredoxin. Leptomycin B, an antifungal antibiotic, suppresses the function of the key exportin protein CRM1 and suppresses virtually all nuclear export by interfering with a conserved cysteine residue. This also blocks the cell cycle (Kudo et al., 1999a) which is strongly regulated by shuttling of various cyclin proteins (Jackman et al., 2002). The deacetylase mHDAC6 is actively maintained

in the cytoplasm and nuclear import also arrests cell proliferation (Verdel et al., 2000). Similarly, oxidative stress mediates nuclear localization of HSF1 and its binding to HSP gene promoters (Fedoroff, 2006).

Although the involvement of a conserved cysteine in exportin1 is suggestive from a redox perspective (i.e., could oxidation directly inhibit nuclear export?) general wisdom currently favors that control of cytoplasmic–nuclear transport resides in access to the import or export signatures of cargo. This allows for differential transport of cargos that may have antagonistic actions or that are relevant to different functions or electroplasmic states. Redox control of some cytoplasmic–nuclear transport may also arise from the pervasive involvement of kinase-phosphatase activities (Jans, 1995; Poon and Jans, 2005). Furthermore, all of the MAPK pathways that regulate nuclear transport are also upregulated by oxidizing conditions and inhibition of tyrosine phosphatases. All of this suggests that a major impact of radiation and xenobiotic interactions will involve transport and localization of numerous critical regulatory peptides.

Most transcription factors relevant to the multiple stressor paradigm are regulated by redox-sensitive transport. The nuclear pore complex and nuclear transport also critically regulate apoptosis. Cytochrome *c*, caspase-2, caspase-3, caspase-6 and apoptosis inducing factor are all transported into the nucleus during apoptosis, whereas acetylated histone H2A and inhibitors of apoptosis are exported (Fahrenkrog, 2006). Heat-shock proteins can inhibit import of apoptosis-inducing factor. Export of the glucocorticoid receptor and PKA from the nucleus involves the Ca^{2+}-binding protein, calreticulin, suggesting potential linkages between calcium homeostasis and transport activities. Furthermore the Ca^{2+}-responsive phosphatase, calcineurin binds NFAT4, masking its nuclear export signal (Poon and Jans, 2005). Ca^{2+} elevations are generally associated with the oxidative phase of electroplasmic cycles. Fahrenkrog (2006) suggests that reduced nuclear import, as seen in aging cells or following heat shock, may in fact represent a stress response. Indeed, severe oxidative stress disrupts nuclear protein import (Kodiha et al., 2004).

Release of the glucocorticoid receptor (a key effector of the stress hormone axis) from HSP-90 and subsequent nuclear import are inhibited by oxidative conditions. This traces to Cys-481 (Okamoto et al., 1999). Alternatively, nuclear export and transcriptional inhibition were enhanced when the glucocorticoid receptor was phosphorylated by activated JNK (Itoh et al., 2002). The AP-endonuclease (=Redox factor (1) functions in DNA repair and activation of oxidatively induced transcription factors like AP-1. It also alters drug resistance of cancers. This factor is translocated to the nucleus following oxidative stress in some cell types or may be maintained

in the nucleus by inhibition of export (Jackson et al. 2005). The nuclear localization signal for NF-κB is masked by IκB until IκB is phosphorylated and degraded. IκB itself also shuttles between the nucleus and cytoplasm (Macara, 2001). Nuclear export of telomerase reverse transcriptase traces to phosphorylation of tyrosine 707, and this is effected by H_2O_2 (Haendeler et al., 2003).

This mechanism reduces the anti-apoptotic actions of the telomerase, and has strong implications for telomere maintenance and cell senescence. Antioxidants can reverse these impacts (Furumoto et al., 1998) suggesting that free-radical stress associated with radiation and xenobiotics could accelerate cellular senescence, particularly in humans.

The transcription factor Nrf2 is essential for activating phase II detoxification and oxidative stress defensive genes acting via antioxidant response elements and Maf recognition elements (ARE/MARE). Nrf2 is inhibited when bound in the cytoplasm by Keap1. Previous models envisioned that oxidative stress frees Nrf2 and allows its transport to the nucleus (Hoshino et al., 2000). Velichkova and Hasson (2005) present evidence that Keap1 has a nuclear export motif which is neutralized by oxidative modification of crucial cysteine residues. This then allows the complex to move to the nucleus via a nucleus localization motif on Nrf2. Nrf2/Keap1 are considered to act as a sensor for cytoplasmic oxidative stress. The Bach2 transcription factor is related to Nrf2, but has opposite actions on induction by MARE. Conditional nuclear export of Bach2 is also sensitive to oxidative stress, perhaps via a cysteine residue in the cytoplasmic localization motif (Hoshino et al., 2000).

Growth, Metabolism, PI3K and Aging

Insulin, IGF-1 and all growth factor signaling requires endogenous production of free radicals (mainly by membrane-bound NOX systems). Free radicals and low dose radiation can substitute for receptor ligands to activate NOX, induce growth, or at higher levels, growth arrest and apoptosis. Mitogen-activated protein kinase pathways involved include MAPK-ERK (growth, apoptosis, long-term potentiation), PI3K (metabolism, aging, growth, apoptosis), JAK-STAT (growth hormone, leptin, cytokines), JNK (immunity, stress), and p38 (stress) (see Schmidt-Ullrich et al., 2000; Dent et al., 2003). Of these, radiation biology has tended to emphasize JNK and p38 as stress response pathways, but MAPK-ERK and (especially) PI3K modulate aging processes and maximal longevity. Important impacts of PI3K relating to radiation and xenobiotics include NOX (Frey et al., 2006), the target of rapamycin (mTOR), the multiple drug resistance proteins (e.g. p-glycoprotein and MRP), the chaperone protein gp96, ATM (the mutation causing the progeroid syndrome, ataxia telangiectasia), oxidation of

phospholipids like phosphatidylserine and the dietary-restriction-regulating deacetylase, SIRT1. ATM also induces high sensitivity to radiation. Our previous research demonstrated an inverse relationship between adult body size and intra-specific longevity within rats and mice, and curtailed maximal longevity of giant mice (Rollo et al., 1996; Rollo, 2002). Giant transgenic growth hormone mice, which are a model of upregulated free-radical processes (Lemon et al., 2003; Rollo, 2007) were also more sensitive to radiation (Lemon et al., unpublished).

Mutations in the PI3K pathway that extend longevity, and the longevity enhancement associated with dietary restriction and SIRT1, are tightly regulated by activation of forkhead transcription factors. Activation of FOXO4 (=AFX) is suppressed by PI3K activity and phosphorylation by PKB/Akt. FOXO4 is activated following nuclear import but CRM1 exports the peptide. Phosphorylation of FOXO4 by PKB actually occurs in the nucleus and inhibits return transport of the exported transcription factor (Brownawell et al., 2001). Phosphorylation of FOXO4 by PKB/Akt also creates two 14-3-3 binding motifs. Binding of 14-3-3 to both of these sites prevents FOXO4 DNA binding. 14-3-3 peptides can mask transport signaling motifs on target proteins. Examples include CDC25 phosphatases, telomerase, histone deacetylase and FOXO transcription factors (Obsil et al., 2003). For FOXO1, binding by 14-3-3 in response to phosphorylation masks a nuclear localization signal, preventing nuclear import (Zhao et al., 2004).

DNA Modifications, Repair and Regulation

A new wrinkle in redox regulation was the identification of an oxidative "hotspot" in the promoter regions of the vascular endothelial growth factor gene associated with iron residues. Specific modification of guanine in the hypoxia inducible factor-1 response element enhanced incorporation of Hif-1 and Ref-1 into the transcription complex and doubled transcription of a reporter gene (Ziel et al., 2005). This implies that DNA sequences may be adaptive regulatory targets of oxidative signaling which is potentially reversible via DNA repair systems. Such a mechanism resembles a re-writable memory device.

The General Proteomic Code

Regulatory proteins are tuned by a multiplicity of mechanisms, including chaperones, ligands, dimerization, scaffolds, acetylation (p300/CBP, SIRT, HDAC), phosphorylation (kinases, phosphatases), methylation, oxidation, nitrosylation, ubiquitination-degradation, glycosylation, pH, voltage (voltage-gated channels), and transport-compartmentalization. Some of these mechanisms

have been hailed as comprising a new "Histone code." In fact this extends to regulatory peptides throughout the cell and should consequently be considered as a more general "Proteomic Code."

Consider p53 regulation. Redox regulation of p53 is well established, but complex. Radiation and oxidative stress can induce transport and p53 activity, but this may partially be an indirect response to DNA damage. Depletion of GSH and treatment with H_2O_2 can inhibit p53 whereas hypoxia is activating (Meplan et al., 2000). A number of antioxidants increase p53 activity and induce apoptosis in several types of cancer (Brash and Havre, 2002). Oxidative status of multiple cysteine residues modifies p53 binding to response elements. When activated by DNA damage, reversible oxidation of the p53 residue Cys277 specifically modulated binding of p53 to response elements of the GADD45 promoter, but did not change p21WAF1/CIP1 binding (Buzek et al., 2002). The ATM protein (PI3K pathway) phosphorylates p53 and it is required for induction of p53 activity by radiation (Meplan et al., 2000). Some variability in p53 binding may also trace to variations in the p53 response element binding motif. Selenium (seleno-L-methionine) induced redox-factor-1 (Ref1) and p53 proteins and specifically upregulated the DNA repair pathway mediated by Ref-1 – p53. The p53 cysteine residues 275 and 277 were implicated. This was protective against induction of DNA damage by UV radiation.

Ref-1 itself has cysteine reducing abilities (Carrero et al., 2000) and it is recycled by thioredoxin (Fedoroff, 2006). Ref-1 functions to reduce sensitive cysteine residues in the DNA binding domain of p53, which is essential for transcription (Meplan et al., 2000). Brca1 was also required for selenium induction of DNA repair pathways (Seo et al., 2002; Fischer et al., 2006). Differential response element binding by p53 could impact major functions such as DNA repair, apoptosis, and cell-cycle regulation. A nuclear export motif occurs in the NH2-terminal region of p53. Phosphorylation following DNA damage can mask this signal (Zhang and Xiong, 2001a). Tetramerization of p53 in the nucleus also masks the nuclear export signal, ensuring nuclear retention until tetramere dissociation (Stommel et al., 1999). Ref-1 promotes p53 tetramere formation and stability. MDM2 is also a key regulator of p53, promoting ubiquitination and degradation in the cytoplasm, and inhibiting transcription in the nucleus. It may also interfere with acetylation by p300/CBP (Zhang and Xiong, 2001b). Alternatively, SIRT1 deacetylases p53 (Finkel and Cohen, 2005). Binding of p53 by 14-3-3 peptide also enhances activity (Meplan et al., 2000). Understanding of p53 regulation remains incomplete, but there is clearly great complexity entailing numerous interacting mechanisms.

Like p53, the critical transcription factor FOXO3 is acetylated at 5 different lysine residues, and phosphorylated at eight serine or threonine residues

(Brunet et al., 2004). Acetylation can involve SIRT1 (Finkel and Cohen, 2005). Nuclear transport proteins may also be both phosphorylated and acetylated (Wang et al., 2004; Poon and Jans, 2005). Interestingly, direct phosphorylation and acetylation (via p300) of Importin α1 was mediated by AMP-activated protein kinase (AMPK). AMPK can mediate a stress signal of low energy supply (Wang et al., 2004). Thus, the numerous mechanisms impinging on various signaling peptides appear to provide mechanisms to obtain combinatorial specificity across a potentially broad spectrum of targets. Multiple stressors have enormous potential to rattle this cage.

Histone Deacetylases (e.g. SIRT) – Histone Acetylases (p300/CBP) and Regulation of Chromatin Structure, Epigenetics and Gene Transcription

Intense interest in sirtuins (NAD^+-dependent deacetylases) emerged with the recognition that sirtuins (SIRT) regulate responses to dietary restriction associated with extended longevity. Actions of SIRT involve the forkhead (FOXO) transcription factor that is upregulated when Akt/PKB (i.e. PI3K pathway) is downregulated. Besides acting on chromatin histone proteins to inactivate gene transcription (via formation of heterochromatin), SIRT deacetylases critical regulatory factors including Ku70, p53, NFκB, PCG1-α1, and PPARγ (Finkel and Cohen, 2005; Frankel and Rogina, 2006). Sirtuins extend longevity of yeast, nematodes and *Drosophila* (Wood et al., 2004; Frankel and Rogina, 2006). Resveratrol and some other flavonoids upregulated SIRT, mimicking the DR response (Wood et al., 2004). Decreasing another deacetylase that is not NAD^+ dependent, upregulated SIRT and extended fly longevity (Frankel and Rogina, 2006).

Despite intense interest in SIRT there is little reciprocal gerontological discussion of acetylases that must be equally important in modulating longevity and the DR response. Indeed p300/CBP are important acetylases that are likely antagonists of SIRT1 and other HDAC. Like SIRT1, p300/CBP also modify important transcription factors like p53 and NFκB. Oxidative conditions may promote histone acetylation, perhaps acting via these pathways (Gilmour et al., 2003). Interestingly, the longevity of *Drosophila* was extended by ~40–50% by pharmacological inhibition of deacetylases which leads to increased histone acetylation (Kang et al., 2002). Feeding adult flies 4-phenylbutyrate (PBA), a histone deacetylase inhibitor, significantly extended longevity without impacting locomotor vigor, reproduction or stress resistance. Histone protein acetylation was globally elevated and gene expression was complexly altered (including upregulation of SOD, GSH-S-TR, Cytochrome P450, 3 chaperones. Downregulated genes included glyceraldehyde-3-phosphate dehydrogenase, NADH: ubiquinone reductase, cytochrome oxidase subunit

VIb, fatty acid synthetase and cyclin-dependent kinase (Kang et al., 2002). These authors suggest (like Droge, 2005), that extension of longevity may require optimal balance between expression and repression of various alternative gene sets regulated by changes in chromatin structure.

Compensation for environmental or genetic stress (e.g. toxins, temperature, mutations) may involve developmental, molecular, physiological, behavioural or maternal adjustments, even within isogenic inbred strains (Crawley, 1996; Gingrich and Hen, 2000). Chromatin can transmit heritable non-genetic variation via alterations in DNA methylation and associated histone protein structure. Such mechanisms can fix the lifetime metabolic character of young mice (Cooney et al., 2002; Jaenisch and Bird, 2003; Rollo, 2006) and may even suppress transgenic insertions via local induction of heterochromatin (Morgan et al., 1999). Richardson et al. (2006) suggest that exposure of neonatal mice to dieldrin may cause epigenetic fixation of elevated NURR1, a transcription factor that regulates dopamine transporters, resulting in greater susceptibility of the dopamine system to various toxins in later life.

Demethylating actions of radiation have some potential to release transposable elements or viruses that are locked down by embedding them in heterochromatin. Indeed, inhibition of histone deacetylases can induce expression of bovine leukaemia virus (Merezak et al., 2002). Release of transposons theoretically could explain some genomic instability induced by radiation.

Heat-Shock Proteins: Roles in Stress, Immunity, Development and Cell Function Relative to Radiation and Multiple Stressors

Chaperone proteins function in housekeeping activities including protein folding, protein transport across membranes, normal protein turnover, and transcriptional protein assembly. They also critically contribute to important signaling mechanisms, including those involved in cell senescence and cell death (Soti et al., 2003; Bagatell and Whitesell, 2004). Numerous heat-shock/chaperone proteins are upregulated as part of the generalized stress response which includes induction by heat-shock and oxidative conditions (e.g. HSP70) (Callahan et al., 2002). Fedoroff (2006) differentiates between the cytoplasmic stress response and the endoplasmic reticulum "unfolded protein response." The latter stress response is triggered by the presence of misfolded or unfolded proteins. This involves elevated Protein disulfide isomerase activity (which acts on disulfide bonds) and associated GSH depletion. The actions and effectiveness of HSPs determines cell function, recovery and survival. Thus, HSPs are critical players in a multiple stressor paradigm. HSPs increase xenobiotic resistance, including resistance to drugs,

and many (e.g. HSP70 and HSP90) are strongly anti-apoptotic (Soti et al., 2003; Bagatell and Whitesell, 2004). HSP70 may be activated by glutathionylation at sensitive cysteine residues and the coordinating transcription factor HIF1 is directly regulated by redox (Fedoroff, 2006).

Profound effects of stress proteins on immune function have long been appreciated, but mechanisms have only resolved recently (Pennell, 2005). Gp96 (=glucose-regulated protein 94), a member of the HSP90 family, is a chaperone protein that occurs in the endoplasmic reticulum and cell membrane. It functions in binding and processing antigens and proteins and effectively presenting them to the immune system (e.g. Arnold-Schild et al., 1999; Gidalevitz et al., 2004; Binder and Srivastava, 2005; Srivastava, 2005; Biswas et al., 2006). A major source of proteins transported to the endoplasmic MHC system are appropriately-sized fragments derived from ubiquitin-proteosome function. These are transported into the endoplasmic reticulum by the "transporter associated with antigen processing" (TAP). A "presentasome" complex involving the proteosome, TAP and heat-shock proteins is suggested. MHC I molecules loaded with protein are then transported to the cell surface by way of the golgi (Castellino et al., 2000; Binder et al., 2001; Norbury et al., 2004). Gp96 elicits $CD8^+$ T-cell responses against its bound peptides, which requires access to the MHC cross-presentation pathway of antigen presenting cells (dendritic cells, macrophages, B lymphocytes). Neutrophils and monocytes that encounter gp96 that is shed following apoptosis bind the protein at the site of apoptosis (Radsak et al., 2003).

Antigen-presenting cells likely migrate to lymphatic organs to prime T-cells. This may involve release of HSP such as HSP70 (Kumaraguru et al., 2003). HSP may also exit normal cells and induce potent immunological effects (Asea et al., 2002). This can involve secretion of small membrane vesicles (exosomes) that are produced by many cell types. Many other proteins are also found in exosomes (Lancaster and Febbraio, 2005). Toll-like receptors (TLR) are implicated in many of these phenomena, and CD14 is a required link between HSPs and TLR Asea et al., 2002). Mobility of gp96 in the cell membrane may be regulated in part by cytokines (Stolpen et al., 1988). Radiation can induce an inflammatory environment, including up-regulation of heat-shock proteins (Friedman, 2002). Gp96 increases after irradiation in treatment of cervical cancer, and resistance to radiotherapy was associated with its expression (Kubota et al., 2005). This suggests that inhibition or suppression of this chaperone might enhance radiation effectiveness. Alternatively, radiation and vaccination with gp96 from viral-infected cells killed by radiation may synergistically inhibit tumor cell growth (Liu et al., 2005b).

Barley leaf shows large increases in the mRNA for a heat-shock protein of the HSP90 family after exposure to powdery mildew. The protein shows

remarkable similarity to vertebrate glucose–regulated protein 94 (=gp96) (Walther-Larsen et al., 1993). As in mammals this protein is involved in immunological responses, including the hypersensitivity response of plants (which involves an NOX and closely resembles the mammalian bystander phenomenon).

Barley leaf mRNA showed strong increases in the mRNA of a heat-shock protein of the HSP90 family, gp94, in response to infection with powdery mildew. The encoded protein closely resembles vertebrate glucose-regulated protein 94 (=gp96) (Walther-Larsen et al., 1993).

Thus, the association of gp96, NOX and immunity is phylogenetically ancient. In mammals, HSP receptors include TLR, CD91 and scavenger receptors type A, LOX-1, CD94, CD40, CD36 and CD 14 receptors (Binder and Srivastava, 2004, 2005; Quintana and Cohen, 2005; Biswas et al., 2006; Warger et al., 2006). CD91 is considered the major sensor of danger (McBride et al., 2004), and high CD91 expression is the only known marker for HIV resistance (Stebbing et al., 2005). A role for CD91 was contested (Berwin et al., 2002), but the weight of the evidence strongly supports a role of CD91 as a primary receptor for gp96 (Binder and Srivastava, 2004; Srivastava, 2005). Peptides introduced to the cytosol were processed 100 times more efficiently (to major histocompatability complex I molecules) when bound by gp96 or HSP70 (as opposed to free peptides) (Binder et al., 2001). A cytokine function of gp96 was questioned based on the possibility that earlier experiments were performed with materials contaminated with microbial products (e.g. Tsan and Gao, 2004a,b). HSPs, in fact, may have little independent ability to activate the immune system but they bind diverse ligands (including polyliposaccharide) and greatly enhance detection and signaling of immunological receptors (Quintana and Cohen, 2005; Warger et al., 2006). Regardless, preparations from systems unlikely to have microbial contamination support some role of HSP to stimulate macrophages and dendritic cells (Quintana and Cohen, 2005). Thus, gp96 enhances immune responses in a wide variety of situations.

Remarkably, mice vaccinated with gp96 derived from tumor-, virus or bacteria-infected cells developed T-cellular immune responses with corresponding specificities (Rapp and Kaufmann, 2004; Demine and Walden, 2005). In some patients, tumor-derived gp96 peptide complexes strongly suppressed metastatic melanoma (Belli et al., 2002). Specific immunity to tumors induced by gp96 (and little such function in HSP90), may trace to the ATPase activity of gp96, which may be required to transfer proteins to acceptor molecules (Udono and Srivastava, 1994). Anti-tumor immunity provided by gp96 isolated from sarcoma cells was lost of the CD91 receptor was down regulated (Binder et al., 2004). Thus, gp96 is of great interest as a contributing component of vaccines (Liu et al., 2005b; Srivastava, 2005). Given a strong

role in immunity and recognition of foreign elements, it is significant that gp96 is closely associated with the membrane-bound NAD(P)H oxidases that serve in immunological responses in mammals. A common denominator for mechanisms inducing HSP70 is oxidation of the cytosol. Altered protein structure by modification of cysteine residues has been proposed for HSP70 as this mechanism was also found in HSP33. Changes in activity of heat-shock proteins in response to oxidative conditions suggests that immunogenicity may also be redox sensitive (Callahan et al., 2002).

Cellular stress responses may be considered with respect to various compartments, and the endoplasmic reticulum is strongly targeted by stress because it is the site where protein folding essential to proper enzyme functions takes place. Disruption results in accumulation of misfolded proteins (Fathallah-Shaykh, 2005). Glioma cells that are resistant to oxidative stress show upregulation of a connected system of genes that includes gp96 and many associated with NOX activity (e.g. GSH-S transferase pi, peroxiredoxin 1, thioredoxin reductase 1, GADD34). Important connections in this system include Keap1, Nrf2, and ARE response elements (Fathallah-Shaykh, 2005). This process involves the actions of various chaperone proteins. Chaperone proteins rarely act alone but are normally associated with larger protein complexes (Bagatell and Whitesell, 2004). Thus, gp96 is closely associated with the NOX /oxidoreductase complex on mammalian cell membranes (Scarlett et al., 2005).

Robert et al. (1999) detected membrane surface gp96 on several types of cancer cells and on various immunocytes across vertebrate phylogenies. Surface expression of many HSP, including HSP 60, HSP70 and HSP90 are abundantly expressed in various cancers (Robert et al., 1999; Shin et al., 2003). Robert et al. (1999) suggest it acts as a "danger" signal. A possible wider distribution appears likely if gp96 is commonly associated with NOX. Given the association of NOX with responses to various pathogens and parasites (perhaps best explored in the plant hypersensitivity response) it seems possible that gp96 serves as a component of a sensing and recognition system? In plants, HSP90 members have indeed been added to the list of proteins involved in surveillance and resistance protein-triggered immunity (Schulze-Lefert, 2004). Most interest has focused on cytosolic HSP90.2. The developing paradigm envisions a complex that engages ATP-dependent modifications of a steroid protein, making it accessible to activating ligand.

Important clients of HSP90 include receptor tyrosine kinases (including IGF-1_r), SRC family kinases, serine/threonine kinases (including Akt/PKB), cell cycle G2 checkpoint kinases, steroid hormone receptors (glucocorticoid, androgens, estrogen, progesterone), and transcription factors (including p53, NFκB) (Bagatell and Whitesell, 2004). HSP90 also serves many other clients functioning during development. Consequently, deflection of HSP90

service to manage stress responses (i.e., protect proteins from misfolding) may de-stabilize development. HSP90 can serve as a buffer against even inherent genetic variation to provide greater canalization of the phenotype (Rutherford and Lindquist, 1998; Queitsch et al., 2002; Rutherford, 2003). Stress that impacts HSP90 function may consequently release hidden genetic variation that would facilitate evolutionary responses. It can also release expression of mutations that are otherwise silenced by chaperones (Soti et al., 2003). Functioning of HSP90 is dependent on ATP, so energy depletion, which is reliably associated with cellular stress, would also be destabilizing. Soti et al. (2003) describe the "protein homeostasis hypothesis" which proposes that cellular homeostasis depends on an appropriate match between levels of damaged proteins and other clients versus the complement of chaperones available to serve them. Multiple stressors can be expected to disrupt such balance.

Alternatively, heat-shock proteins may facilitate tumorigenesis by acting as a buffer against the genetic and functional instability associated with cancer (Bagatell and Whitesell, 2004). Under stress this could allow expression of genetic variation that would allow cellular adaptation of cancer lineages. This could extend to genetic instability induced by radiation or xenobiotics. Heavy metals also elicit heat-shock responses and this might be expected to be synergized by radiation (Bagatell and Whitesell, 2004). HSP90 is also of interest in cancer since it stabilizes clients that might be targets for therapy. In breast cancer these include mutant p53, estrogen receptor, and Akt (PKB) (Beliakoff and Whitesell, 2004). Regulatory mechanisms impacted by HSP90 extend to regulation of chromatin structure (Sangster et al., 2003; Sollars et al., 2003) suggesting consequences on release of silenced genes, transposons, viruses or epigenetic alterations.

Professional antigen-presenting cells (monocytes and dendrites) show specific binding of gp96 and specific receptor-mediated internalization of the HSP and bound proteins. Internalized protein colocalizes with surface MHC class I molecules (Arnold-Schild et al., 1999). Gp96 greatly increases the efficiency of presentation of associated peptides to T-cells. As little as 1–2 ng of peptides complexed to gp96 are sufficient to elicit a cytotoxic +independently of binding proteins, and this was associated with release of TNF-α and IFN-γ (Baker-LePain et al., 2004). The powerful role of gp96 in delivering proteins and mediating uptake by professional antigen-presenting cells, makes it especially important that stressed or otherwise defective cells that overexpress gp96 are effectively cleared by engulfing macrophages, since the contents of such cells represent a complex soup of bystander signals to nearby cells and the immune system. Gp96 (and HSP70) remains functional in vitro and complexes formed with peptides in cell-free serum are immunologically active with respect to generating anti-tumor and $CD8^+$ cytolytic T lymphocyte responses (Blachere et al., 1997;

Baker-LePain et al., 2004). In vivo, failure to clear such cells may mediate strong local inflammatory states with the possibility of self-perpetuating vicious feedback cycles. Heat-shock proteins (including HSP10, HSP70, HSP90, calneticulin and gp96) that are released by necrotic cell rupture, are recognized as "danger signals" that may promote inflammation and contribute to bystander cell death. HSP in serum promote maturation of dendritic cells, activation of NF-κB, and subsequent release of inflammatory cytokines (Basu et al., 2000; Soti et al., 2003).

The potential importance of such processes is likely to be overlooked in cell culture approaches to phenomenon like the bystander effect. Indeed, there appear to be two broad potential classes of bystander mechanisms. Activation of the immune system can impact organ to organismal-level responses, particularly via cytokine feedback to central regulation. An endocrinological triumvirate has been proposed as the key organismal regulatory framework. This involves the antagonistic regulation of the stress hormone (waking) and growth hormone (sleeping) axes largely integrated in the hypothalamus, and feedback of status in the periphery from what amounts to a distributed endocrine axis.… the immune system. The immune system is the only other tissue that produces nearly all hypothalamic and pituitary regulatory peptides. The fact that radiation induces the stress hormone axis suggests that peripheral free-radical processes may be sensed and relayed to the hypothalamus (see Rollo, 2002). Even in a cell-free context, gp96 can bind proteins can possibly enhance immunological recognition and activation. Radiation or xenobiotic impacts that overwhelm cell clearance mechanisms could induce a secondary immunological response, mediated partially by release of gp96 and other chaperones that constitute "danger signals" (Soti et al., 2003). This extends to radionucleotide-induced bystander effects as well (Xue et al., 2002). Such a mechanism qualifies as a type of bystander response. Plant responses to pathogens also likely trace to diverse foreign molecule recognition by ligand–receptor interactions and transduction of recognition to responses, including the hypersensitive response (Nimchuk et al., 2003).

Alternatively, local mechanisms that activate associated bystander cells surrounding sites of impact may be relatively independent of the immune system. Thus, P450 monooxidase enzymes involved in detoxification can actively generate free radicals in cell-free media (Clejan and Cederbaum, 1992) and would likely synergize actions of active NOX components in conveying oxidative bystander signals. … in particular the activation of NOX on cell membranes of intact bystander cells (Rollo, 2006). A crucial property of the NOX is that it is activated by free radicals and consequently can maintain free-radical generation via an auto-stimulatory feedback loop (Cai, 2005). Chaperones may also convey direct signals to surrounding cells that sensitize stress and cell death programs (Soti et al., 2003). A remarkable

phenomenon resembling a bystander effect is associated with cells expressing a senescent phenotype. The cell senescence phenotype can be derived by numerous mechanisms including free-radical stress (i.e., related to radiation, organismal aging or xenobiotics). Senescent fibroblasts secrete numerous materials including cytokines, growth factors, degradative enzymes and extracellular matrix. Some factors secreted from senescent cells stimulate the growth of premalignant and malignant (but not normal) epithelial cells (Krtolica et al., 2001). Thus, radiation, pollutants and aging may predispose to both transformation and senescence, and senescence may not provide a defense against cancers as such cells accumulate with age.

The Phosphatidylserine "EAT ME" Signal and Clearance of Defective Cells

A critical aspect of gp96 (and other chaperones) related to radiation, is that heat-shock proteins and their bound client proteins are released or shed from apoptotic cells (Basu et al., 2000). Shedding of active chemical species extends to other important factors such as protein kinase C, phosphatidylserine, arachidonic acid, ATP, cytochrome c, lipid peroxidation products and oxidized sterols. Hydrogen peroxide generated by apoptotic cells is sufficient to induce apoptosis in surrounding cells (Milan et al., 1997; Reznikov et al., 2000; Cusato et al., 2003) and has been suggested as a major bystander candidate (Pletjushkina et al., 2005, 2006). Extracellular ATP can promote cell death acting via purinergic receptors and Ca^{2+} elevations. Ca^{2+} itself is implicated in bystander impacts (Budd and Lipton, 1998; Vinken et al., 2006). Growth factors and cytokines released into serum also have particularly potent inflammatory actions (Zheng et al., 1991; Proskuryakov et al., 2002). Uric acid produced from catabolism of DNA and RNA from dying cells is also a very strong danger signal and immunological activator (Shi et al., 2003). Excitotoxic neurotransmitters can also stress neurons adjacent to those undergoing cell rupture. There is clearly considerable complexity. Add to this that Bauer (2002) distinguishes bystander impacts mediated by gap-junctions versus inter-cellular signals that did not require gap junctions.

Removal of apoptotic cells by phagocytic engulfment may serve to prevent release of cellular constituents, including self antigens that could induce autoimmune responses (Proskuryakov et al., 2002; Fadeel, 2003; Fadeel and Xue, 2006). Clearance of apoptotic cells may induce little or an altered immune response compared to cell rupture (Mevorach et al., 1998). Thus, clearance constitutes an essential aspect of tissue homeostasis which usually highlights only mitosis and apoptosis. Indeed accelerated apoptosis and impaired clearance of apoptotic material exacerbates autoimmune responses in mice (Denny et al., 2006). Furthermore, strong exposure to irradiation-induced apoptotic cells generates auto-antibody production, including anti-cardiolipin and anti-double strand DNA antibodies (Mevorach et al., 1998).

Cardiolipin is a critical and predominant constituent of the mitochondrial membrane. Its oxidation during apoptosis is important with respect to associated cytochrome *c* (Tyurina et al., 2006). In T-cell lineages, ionizing radiation induced apoptosis in association with elevated levels of mRNA and the Apo2 ligand (also called TRAIL). This also activated the AposL death receptor 5 (also called KILLER) associated with apoptosis and externalization of phosphatidylserine. Bax, induced by p53 is also a mediator of apoptosis in irradiated cells (Gong and Almasan, 2000).

Impaired engulfment of apoptotic cells was found in mice with defective lactadherin (=milk fat globule epidermal growth factor 8), a factor produced by activated macrophages that mediates phosphatidylserine recognition. Injection of the mutated protein also induced autoimmunity, probably by masking recognition of phosphatidylserine (Asano et al., 2004; Fadeel and Xue, 2006). Autoimmunity was also obtained in mice overexpressing gp96 on the cell surface, and this involved the MyD88 adaptor protein crucial to TLR signaling (Liu et al., 2003, 2005a). Massive apoptosis was detected in lymphoid organs in a mouse model of multiple sclerosis (with depletion of B and T cells). Injection of apoptotic thymocytes exacerbated demyelination and disease progression (Tsunoda et al., 2005). Activation of NOX by phorbol myristate in HL-60 cells generated SOR, depleted intracellular GSH and peroxidized all three major classes of membrane phospholipid (phosphatidylcholine, phosphatidyl-ethanolamine, and phosphatidylserine). Radiation induced similar changes in rodent brain (Richards and Budinger, 1988). Enteropathogenic infection by *Escherichia coli* also induces externalization of both phosphatidylserine and PKC (crucially involved in NAD(P)H oxidase activation) (Fadeel and Xue, 2006). Activation of PKC involves movement to the cell surface and binding with phosphatidylserine. PKC externalization was also induced by phorbol myristate acetate and ultraviolet light (~25% of cell content) (Crane and Vezina, 2005). Both of these factors also induce NAD(P)H oxidase. Phosphatidylserine also induces NOX, in cell-free media, although its oxidation state was not considered (McPhail et al., 1993).

Apoptosis is associated with externalization of oxidized phosphatidylserine that serves as a signal for ingestion of cells by macrophages (Arroyo et al., 2002). HSP70 and Hsc70 interact with phosphatidylserine, and accelerate apoptosis, an effect exacerbated by ATP. HSP are also able to induce ion channels (Arispe et al., 2004) which could contribute to NADPH channel associations or apoptotic processes. Thus NOX is associated with both apoptotic and clearance functions. Extracellular ATP can induce thymocyte apoptosis via purinoceptor activation. This included early phosphatidylserine externalization, mediated by activation of the cationic PsX7 (=P2Z) channel. Phosphatidylserine movement required ATP-induced Ca^{2+} and/or Na^{+} influx (Courageot et al., 2004). Apoptotic cells express elevated levels of

oxidized phospholipids that function as immunological and pro-inflammatory signals. Fluorescence-conjugated annexin V specifically binds externalized phosphatidylserine, making it a reliable biomarker for apoptosis (Fadeel and Xue, 2006).

Apoptotic cells induced production of T helper cell Th1 and Th2 cytokines, and induced monocyte adhesion in endothelial cells. The latter was inhibited by an antibody specific to oxidized phosphatidylcholine (Chang et al., 2004). Lysophosphatidylcholine can act as a chemoattractant that guides macrophages to sites of apoptotic lesions (Fadeel and Xue, 2006). Oxidized phospholipids induced monocyte adhesion in endothelial cells via MAPK pathways and activation of cytosolic phospholipase A_2 and 12- lipoxygenase (Huber et al., 2006). Externalization and oxidation of phosphatidylserine in the cell membrane is a critical signal to macrophages for engulfing apoptotic cells (Kagan et al., 2003). Oxidized phosphatidylserine externalized during apoptosis is recognized by specific macrophage receptors. Selective oxidation of phosphatidylserine precedes externalization and oxidation is required for engulfment of apoptotic cells. H_2O_2 was effective in oxidation and externalization of phosphatidylserine (Tyurina et al., 2004). Lipid antioxidants (Tyurina et al., 2004), NOX inhibitors, superoxide dismutase and catalase protected all phospholipids from oxidation and externalization of phosphatidylserine (Arroyo et al., 2002). Radiation induces caspase 3 and caspase 8 (Gong and Almasan, 2000). Caspase 3 is redox-regulated and appears pivotal in determining whether cell death proceeds by apoptosis or necrosis. Inhibition of caspase 3 yields necrosis and failure to externalize oxidized phosphatidylserine. Both forms of cell death may be elicited by radiation (Coelho et al., 2000). A complication is that NOX ROS production could inhibit activity of redox-sensitive caspase 3 (Arroyo et al., 2002).

Despite considerable evidence that failure to clear apoptotic cells may induce autoimmunity, and that radiation can exacerbate this process, radiation is not reliably associated with autoimmune responses. The immune system is particularly impacted by radiation, such that various autoimmune diseases can actually benefit from irradiation. Suppression of antigen-presenting dendritic cells and inhibition of the proteosome (which produces peptide fragments of appropriate size for major histocompatability complex processing) are crucial mechanisms of radiation-induced hypo-immunity (McBride et al., 2004). Dosage is likely a crucial factor. Regardless, cell death, clearance and immune responses remain potentially critical for a radiation and a multi-stressor paradigm because high doses or interactions that are additive or multiplicative may shift apoptosis and effective clearance to necrosis. Xenobiotic impacts synergized by relatively low levels of radiation may derive massive cell death without the immuno-suppressive impacts of high dose radiation. This would likely result in clearance failures, immunological infiltration and

inflammation. The enhanced induction of immunological sensitivity (e.g. by gp96) extends a linkage between multiple stressors (including xenobiotics) to allergenic and autoimmune responses.

Multiple Drug Resistance Proteins (MDRP)

Multiple drug resistance proteins (members of the ATP-binding cassette (ABC) transporter superfamily) such as multiple drug resistance protein-1 (MDR-1 gene, P-glycoprotein, Pgp) and multiple resistance protein-1 (MRP-1) export cellular detoxification products, xenobiotics and various conjugates of waste products or toxins. Powell and Abraham (1993) suggest they also excrete growth factors that can mediate strong immunological and stress responses (e.g. FGF, TNF, TGF-α). Steroid hormones may also be exported (Tatsuta et al., 1992). MDR-1 is transcriptionally upregulated by heat stress, heavy metals, drug treatment and UV radiation (Toone et al., 1998). At least 50 family members have been identified (Minier et al., 1999). They can also inhibit apoptosis induced by TNF, serum withdrawal, Fas ligand, and UV radiation (Baker and El-Osta, 2004). MDRP are intensely studied because they can export antibiotics and cancer drugs, resulting in resistance to diverse chemotherapeutic agents. They can also mediate pesticide resistance (Minier et al., 1999). Thus, most medical research aims to inhibit MDRP activity (e.g. Tan et al., 2000). Although MDRPs may be detrimental to cancer therapy and pest control, their activities would be highly advantageous for resistance to multiple pollutants. Unlike MDR-1, MRP-1 mainly transports anionic Phase II-conjugates, and this is highly dependent on high intracellular GSH levels. There are at least 6 MRP homologues (Renes et al., 2000). Multiple drug resistance proteins, in conjunction with p450 enzyme activity, may provide xenobiotic barriers in the kidney, buccal cavity, gastrointestinal tract, endothelium, liver, placenta, blood brain barrier (where they may exclude antidepressants) and choroid plexus (Tatusta et al., 1992; Minier et al., 1999; Renes et al., 2000; Uhr et al., 2000; Miller et al., 2002; Leggas et al., 2004). MDR1 may play a role in host-bacterial interactions in the intestinal epithelium (Ho et al., 2003).

Many elements exported by MDRPs are conjugates of GSH (catalyzed by glutathione-S-transferases), or even GSH itself, indicating a crucial role in detoxification and function of the GSH system. High levels of GSH can downregulate expression of MRP-1, although GSH depletion is not necessarily upregulating (Renes et al., 2000). Zaman et al. (1995) found that GSH depletion impacted MRP-1 more than MDR-1. Renes et al. (2000) suggests that a critical level of GSH depletion may be necessary before MRP-1 transcription is affected. This is consistent with my argument that the lack of obvious dosage response for low dose radiation is that it may

require >50% depletion of GSH to reach a critical threshold for redox vulnerability (Rollo, 2006). In addition, export of oxidized glutathione (GSSG) by MRP-1 (Leier et al., 1996) suggests a mechanism for regulating intracellular redox balance.

Export of ATP by MDRP has potentially profound impacts, including control of endothelial function, apoptosis, the cell cycle, signal transduction, thiol protection and calcium levels. MDRP transport of ATP may be an initial response to radiation. ATP was promptly released by 0.1 Gy of radiation (Powell and Abraham, 1993). Extra-cellular ATP fuels diverse cell membrane and extra-cellular processes, including ion channel and NOX activities. ATP can act on a variety of purine and adenosine receptors (e.g. P1, P2, A1, A2) which may have bystander impacts or derive intracellular auto-stimulatory feedback loops (Powell and Abraham, 1993). The adenosine neurotransmitter system has widespread important impacts, especially in brain. Further, adenosine receptors A_{2B} interact with melatonin via adenylate cyclase to entrain expression of the circadian clock gene Period, thus coordinating the circadian clocks of peripheral tissues like the pituitary with the hypothalamic suprachiasmatic nucleus (von Gall et al., 2002). The circadian rhythm may constitute a large amplitude electroplasmic cycle.

Overexpression of COX-2 (which generates prostaglandins from arachidonic acid) enhances expression of MDR-1 (Sorokin, 2004). NOX (also activated by arachidonic acid) also upregulates MDR-1, as does radiation and heat shock (Minier et al., 1999). Alternatively, MDR is associated with increased expression and activity of CYP3A (a major drug detoxifying cytochrome) (Schuetz et al., 2000; Baron et al., 2001). The association with the membrane NOX is noteworthy as this oxidase is upregulated by radiation (Narayanan et al., 1997; Azzam et al., 2002) and regulates MAPK signaling. Moreover, the oxidase may be part of a larger NADPH oxidoreductase complex that functions to maintaining intracellular ionic and oxidative homeostasis via regulation of general cation and K^+ channels (Scarlett et al., 2005). The MRP-1 gene contains an antioxidant response element and multiple drug resistance is strongly upregulated by free radicals and oxidation (Renes et al., 2000; Sorokin, 2004). Mechanisms generating cellular free radicals induce gene expression for MRP-1 and the rate-limiting enzyme for GSH synthesis, γ-glutamylcysteine (Renes et al., 2000). MRP-1 modulates dauer (diapausing larvae) formation in *C. elegans*, a mechanism associated with modulation of longevity and linked to the insulin/IGF-1 PI3K pathway (Yabe et al., 2005). This pathway has been highlighted in modulation of aging and stress resistance across broad phylogenies spanning yeast, nematodes, insects and vertebrates.

Although MRP-1 is upregulated by free radicals, it does not directly provide protection from radiation or other sources of free-radical damage.

However, drug resistance transporters can export materials that would otherwise exacerbate free-radical stress. A crucial question then is to what extent low-level radiation might upregulate MDR-1/MRP-1 and the shedding of pollutants, and at what dosages possible benefits exceed the possible costs induced by radiation exposure. Demonstrated expression of MDRPs in filtering species such as sponges and some bivalves (gill) suggests a sensitive biomarker for general environmental pollution (Minier et al., 1999) and possibly associated radiation background. The antibody for human P-glycoprotein is effective across broad phylogenies. Interestingly, heavy metal export is one target of these transporters, and export of such materials could reduce free-radical damage (including that induced by radiation). It should also be noted that one client of MRP-1 is leukotriene C_4, a glutathione-conjugated organic anion that functions as an inflammatory mediator (Leier et al., 1996; Renes et al., 2000) and that may be a candidate bystander signal. Alternatively, leukotriene B_4 stimulates release of arachidonic acid which promotes free-radical generation by NOX. This also occurs in cell-free serum (Brash, 2001; Cherny et al., 2001). In fact, activated phagocytes export arachidonic acid, and this can cause free-radical generation on other cells (DeCoursey, 2002). Thus, arachidonic acid qualifies as a bona fide bystander signal in its own right. Arachidonic acid also impacts a number of ion channels (Brash, 2001). TNF-α also upregulates MRP-1, possibly via its ability to generate free radicals (Renes et al., 2000). TNF is also upregulated by radiation and NAD(P)H: quinone oxidoreductase-1 (NOQ1) is a critical component of the TNF signaling cascade to NF-κB. NQO1 is also known to be induced by xenobiotics, oxidants and radiation (McBride et al., 2004; Ahn et al., 2006).

The promoter region of MDR-1 contains a CpG methylation sequence, and the gene is importantly regulated by epigenetic alterations of chromatin structure. It is possible that this gene may be included in early-life epigenetic modifications that effect life-long changes in stress responses. Chromatin regulation involves methyl-CpG-binding protein-2 (MeCP2) and its recruitment of the corepressors, HDAC1 and HDAC2. Gene regulation requires both demethylation of DNA and hyperacetylation of associated histones (Baker and El-Osta, 2004). Since radiation is generally associated with demethylation, this could represent one mechanism of radiation-induced MDR-1 activation.

Toll-like Receptors

TLRs underpin the ability of innate immunity to discriminate between highly diverse pathogens and self. TLRs activate macrophages in response to pathogens which in turn present pathogen-derived peptides to T-helper cells. Of relevance to the free-radical elevations associated with radiation

and p450 xenobiotic detoxification systems, TLR expression (and activation of NF-κB) can also be upregulated by free radicals, including those generated by NOX (Fan et al., 2003; Asehnoune et al., 2004; Qureshi et al., 2006). Expression of TLR4 even confers resistance to hyperoxia-induced lung injury and apoptosis (Zhang et al., 2005; Qureshi et al., 2006). The mechanism of hyperoxia resistance involved the ability to express Bcl-2, a critical element in PI3K-mediated resistance to apoptosis. Lipopolysaccharide induction of free radicals and activation of NF-κB requires direct interaction of TLR4 and NOX4 (Park et al., 2004a). Loss of the TLR signaling element, MyD88, diminished NOX function and killing of gram-negative bacteria (Laroux et al., 2005). TLR4 may also be involved in susceptibility to ozone-induced lung injury (Kleeberger et al., 2000).

TLRs can also act cooperatively in clusters or co-activate via other microbial recognition receptors. Numerous immunocytes employ distinct arrays of TLRs (e.g. neutrophils, natural killer cells, mast cells, B-cells, eosinophils and monocytes). Structure and signaling of TLR closely resemble the IL-1 receptor, activating TRAF6 and NF-κB (Underhill, 2003). Until the 1990s how organisms recognized pathogens was virtually unknown. Since 1991 at least 13 mammalian Toll paralogues (11 expressed in humans) have been found. Each responds to different phylogenetically invariant components of microbial lineages and may have cytosolic or membrane-bound distributions. Like the NOX, TLRs are particularly associated with tissues and locations which face potential pathogenic contacts, including vascular endothelium, bronchial, gastrointestinal and urogenital epithelium and blood-brain barrier (Hopkins and Sriskandan, 2005). Interestingly, most of these are also regions expressing multiple drug resistance proteins. TLR are phylogenetically conserved across plants to insects to vertebrates (Lescot et al., 2004).

TLR2 and TLR4 were implicated as gp96 receptors on immunocytes (Baker-Lepain et al., 2004). HSP, including gp96, complex with TLRs and cooperate in binding and signaling diverse ligands (Underhill, 2003). Earlier concerns that microbial contaminants may have artifactually mediated the apparent gp96 interaction with TLRs have been offset by careful attention to uncontaminated preparations. HSP may have little independent immunogenicity, but even low amounts of gp96 greatly (up to 100-fold) amplified bacterial product-induced TLR-mediated signaling relevant to activation of T cells and dendritic cells (Warger et al., 2006). Membrane expressed TLR2 and TLR4 bind HSP (HSP60, HSP70, HSP90/gp96), suggesting they may serve as immunocyte receptors for HSP. This also suggests the possibility that HSP may be closely connected to surveillance systems more generally. With respect to bacterial lipopolysaccharide, this may be first bound by CD14 associated with TRL4, and then transferred to a HSP70-HSP90 complex (Underhill, 2003).

One outcome of TLR activation is engagement of the NADPH oxidative burst, and ultimately, adaptive immunity (Hopkins and Sriskandan, 2005). Lipopolysaccharide from gram negative bacteria mediates direct activation of NOX4 via TLR4. This is known to involve Rac1. Lipopolysaccharide from *Helicobacter pylori* also induced NOX1 (~$gp91^{phox}$) activity and mRNA via a PI3K pathway activation of Rac1 in gastric mucosal cells. Expression of NOXO1 (~$p47^{phox}$) mRNA was also enhanced. Flagella of *Salmonella* also activated NOX1. Detection of lipopolysaccharide and flagella involved TLR4 and TLR5 respectively (Geiszt and Leto, 2004; Kawahara et al., 2005). Signaling of TLR4 (and many other TLRs) via MyD88 to IL-1R associated kinase to TRAF6 (tumor necrosis factor-associated factor-6) can activate NF-κB (Tsan and Gao, 2004a; Park et al., 2004b). NF-κB activation may also be mediated by free radicals generated by NOX4, since H_2O_2 activates NF-κB (Park et al., 2004a, b). In the colon epithelium, TLR5 mediated free-radical production via NOX1 in response to stimulation by flagellin from *Salmonella enteritidis* or by lipopolysaccharide from *Helicobacter pylori*. In addition to activation of NF-κB, TLRs induce AP-1, TNF-α, IFN-β, IL-8 (involved in chemoattraction of immunocytes) and TGF-β-activated kinase 1 (Yamamoto et al., 2003; Kawahara et al., 2004; Kawai and Akira, 2006).

The IL-1 receptor associated kinase is a key element in TLR signaling, linking receptor activation to TRAF6 and subsequent activation of MAPK (e.g. ERK1/2, p38), NF-κB, TNF-α, IL-1and IL-8. A complex containing HSP90 and Cdc37 regulate the folding and possible level of activation of IL-1R associated kinase, providing a further linkage of HSP90 family members to immunological recognition. In fact, heat-shock proteins are also involved in regulation of many important signaling elements including Raf-1, p53, and IκB (De Nardo et al., 2005). Overall, this discussion identifies complex interactions among NOX, Rac, TLR, HSP90, and gp96. Although TLRs recognize specific pathogen derivatives, they may also respond to other factors. They may monitor endogenous damage, including signals generated by radiation (McBride et al., 2004). TLR9 is activated by unmethylated CpG didioxynucleotides. Mammals have few such sequences, and most are methylated, providing discrimination of bacterial and mammalian host DNA (Hemmi et al., 2000; Takeshita et al., 2001; Chuang et al., 2002).

Although such signals may be associated with recognition of pathogens, it is interesting that demethylation of mammalian CpG islands may release endogenous viral activity, and radiation (and cancer) are generally associated with demethylation. Growing evidence implicates inappropriate responses to host DNA in autoimmune diseases. T cells from lupus patients are hypomethylated, and mice receiving CD4 + T cells that have been chemically demethylated developed a lupus-like condition (Blank and Shoenfeld, 2005; Kaplan et al., 2005). More than 100 genes sensitive to methylation status have been

detected. Upregulation of such genes (particularly performin) may contribute to autoimmunity by altering the regulation of apoptosis and clearance (Kaplan et al., 2005). HSP90 is also involved in signaling induced by CpG motifs (Quintana aand Cohen, 2005). Immunization of mice with DNA from activated lymphocytes induced antibodies against double-stranded DNA, and others characteristic of lupus (Qiao et al., 2005).

TLR9, which is the well-characterized DNA recognition receptor is highlighted in serious conditions such as lupus and multiple sclerosis (Anders, 2005; Means et al., 2005; Prinz et al., 2006). Strong signaling via TLR9 can induce apoptosis and even organ failure (Yi et al., 2006). For lupus, DNA from nucleosomes may be most critical, highlighting the need for efficient clearance of defective cells (Radic et al., 2004; Blank and Shoenfeld, 2005; Ishii and Akira, 2005). Remarkably, nucleosomes may be released from the nucleus to the cell surface during apoptosis. The presence of nucleosome core particles in apoptotic membrane blebs may provide an additional signal for phagocytosis (Radic et al., 2004). Immunization of mice with nucleosomes triggers Th1-type autoimmune T cells (Mohan et al., 1993; Blank and Shoenfeld, 2005). Other host factors may also serve as endogenous ligands, particularly those associated with cell damage or dysfunction (e.g. heat-shock proteins, fibrinogen, hyaluronan, heparin sulfate and mRNA) (Tsan and Gao, 2004a; De Nardo et al., 2005). An important question, however, is whether some such findings are confounded by the presence pathogen contaminants (Tsan and Gao, 2004a,b).

TLR appear to be involved in recognition of DNA damage and NF-κB-associated responses. The cancer-treatment drug "taxol" activates NF-κB and may be a ligand for TLR4. This receptor may also be required to mediate NF-κB activation in response to chemically mediated DNA damage, possibly linking radiation-induced DNA damage and NF-κB activation to TLR (Park et al., 2004a,b). In this regard, low dose radiation (~2Gy/d) was beneficial in follicular lymphoma, and besides upregulation of p53, elevated expression of TLR4 was part of the "immune signature." Induction of specific immune modulators by radiation was suggested to likely impact death and clearance of tumor cells (Knoops et al., 2005).

Specific immunity to tumors by gp96 (and little such function in HSP90), may trace to the ATPase activity of gp96, which may be required to transfer proteins to acceptor molecules (Udono and Srivastava, 1994).

Conclusions

Despite great complexity, some unity amidst diversity can be found in considerations of diverse stressors, including aging, radiation, xenobiotics and many others. This is possible because stressors share a strong basis

in free-radical-mediated regulation and coordination of biological stress response functions. A unifying perspective highlighting integrated electroplasmic cycling brings together diverse mechanisms associated with free-radical processes, growth and development, signal transduction, ion channels, chaperone proteins, immunological activities, clocks, and transcription. Regardless, there remains bewildering complexity, and the intricacies of regulatory mechanisms continue to unfold at a great pace. There is a serious need for a computer modeling and systems approach to provide a concrete focus for research into regulatory circuitry, and some ability to predict the consequences of multiple stressors on ecosystems and the people who inhabit them.

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