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Forum Review Article

Strategies for Evaluation of Signaling Pathways and Transcription Factors Altered in Aging

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ABSTRACT

Aging is characterized by an accumulation of oxidative injury to DNA, RNA, proteins, lipids, and carbohydrates. In addition to damage, oxidative stress can initiate cell signaling cascades that modulate cell function, growth, and death. Aging and two common age-related diseases, diabetes mellitus and atherosclerosis, may share common oxidant-related signaling pathways that lead to abnormal transcription factor activation and ultimately to cellular dysfunction, degeneration, or death. This review will focus on approaches to evaluate key redox-sensitive signaling pathways and the transcription factors altered by diabetes, atherosclerosis, and aging. Antiox. Redox Signal. 2, 379–389.

INTRODUCTION

A PARADOX OF AEROBIC LIFE is that higher eukaryotic organisms cannot exist without oxygen; yet oxygen is inherently dangerous because of the generation of reactive oxygen species (ROS), which are capable of damaging nucleic acids (RNA and DNA), carbohydrates, lipids, or proteins. "Oxidative stress" is associated with a disturbance in the balance between pro-oxidants (ROS, free radicals) and antioxidants in favor of the pro-oxidants (Sies, 1991). The resulting oxidative damage is currently thought to be a pathobiochemical mechanism leading to the initiation or progression of aging, inflammation, and carcinogenesis. In addition to damage, oxidants can initiate cell signaling cascades that modulate cell function. The complex physiological condition of aging as well as two common age-related diseases, diabetes mellitus and atherosclerosis, may all share common oxidant-related signaling pathways leading to abnormal transcription factor activation and ultimately the evolution of a disease process. The complexity of the processes of oxidative injury and the variety of systems affected are beyond the scope of this review. The emphasis of this review will be on approaches used for evaluating key signaling pathways known to be redox-sensitive and the transcription factors altered by diabetes mellitus and aging.

The free radical or oxidative stress theories of aging entail the hypotheses that oxidants cause cellular damage and result in some of the characteristic changes of aging (Ames *et al.*, 1993; Beckman and Ames, 1998). Oxidative damage to DNA, proteins, and other macromolecules accumulate with age and may contribute to degenerative diseases in aging by disrupting cellular homeostasis (Ames *et al.*, 1993; Beckman and Ames, 1998). Damaged DNA

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may be repaired by enzymes that excise the lesions. The altered bases may then be excreted, forming the basis for a number of methods to estimate the degree of oxidative damage. Ames *et al*., estimated that a single human cell experiences about 10,000 oxidative hits per day, and, if lesions are not removed, damaged DNA accumulates with age (Ames *et al*., 1993). Oxidative damage to DNA and other macromolecules then may play an important role in carcinogenesis, cataract formation, cardiovascular disease, and brain dysfunction. Oxidant-induced damage to the mitochondrial genome may be particularly important for two reasons: (1) the ability to repair DNA is limited in the organelle and (2) mitochondria constitute the greatest source of oxidants as approximately 85% of the oxygen used by the cell is consumed by the mitochondrial electron transport chain during the production of adenosine triphosphate (ATP).

Mitochondrial integrity and function are influenced by both aging and diabetes mellitus. Oxidant-mediated repression of mitochondrial gene transcription in hepatocytes has been reported in streptozotocin-induced diabetic rats (Kristal *et al*., 1997), whereas increased mitochondrial gene expression was seen in human skeletal muscle from patients with diabetes mellitus (Antonetti *et al*., 1995). Superoxide ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2) production by mitochondria increases with advancing age in mice (Sohal *et al*., 1994). Excess production of free radicals is associated with increased mutations in mitochondrial DNA or alterations in mitochondrial gene expression, fragmentation of the mitochondrial genome, and programmed cell death or apoptosis (Richter *et al*., 1996; Zamzami *et al*., 1996; Li *et al*., 1997; Yakes and Van Houten, 1997). Two signaling cascades that are integral to the development of apoptosis, cell survival, and cell proliferations are the mitogen-activated protein kinases (MAPK) and nuclear factor-kappa B (NK-κB).

MAPKs

The MAPK cascade consists of three interrelated pathways including the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal/stress-activated protein kinases (JNK/SAPK), and p38 kinases. Activation of each of these pathways is associated with the induction of differentiation, cell survival, proliferation, and/or apoptosis (Xiz *et al*., 1995; Chen *et al*., 1996; Jimenez *et al*., 1997; Bhat and Zhang, 1999). Several phosphorylated substrates for ERK, JNK, and p38 have been identified, most notably members of the early-response proto-oncogene family, including c-Fos and c-Jun. c-*fos* and c-*jun* are early-response genes that are induced by a variety of external stresses and encode protein subunits that can form Jun homodimers or Jun/Fos heterodimers, which comprise the activator protein-1 (AP-1) transcription factor (Angel and Karin, 1991). The AP-1 transcription factor regulates genes involved in cell cycle progression, inflammation, and apoptosis. For example, activation of early response genes is involved in transition from G_0/G_1 into the S phase of the cell cycle (Angel and Karin, 1991). Overexpression of c-*jun* in tracheal epithelial cells is associated with increased DNA synthesis and cell transformation (Timblin *et al*., 1995). A causal effect between expression of c-*fos* and the induction of apoptosis has been documented in Syrian hamster embryo cells (Preston *et al*., 1996). In a recent review, Guyton *et al*. concluded that attenuation of ERK activation by oxidants in hepatocytes from old rats is consistent with the age-related decline in the capacity to respond to both proliferative and stress stimuli (Guyton *et al*., 1998). Interestingly, they found little age-related differences in JNK activation by the same stimuli. However, decreased expression of c-*fos* has been reported in an *in vitro* model of senescent human fibroblasts (Seshadri and Campisi, 1990) and *in vivo* in hearts of aged rats (Tsou *et al*., 1996). More recently, Igarashi *et al*. characterized the effect of hyperglycemia on the activity of p38 mitogen-activated protein in vascular cells *in vitro* and in aorta from diabetic rats (Igarashi *et al*., 1999). High levels of glucose or diabetes were found to activate p38 by a pathway not identical to ERK. The interaction between age-related and glycemia-related effects is in need of further delineation.

NF-κB

NK-κB is a transcription factor encoded by members of the Rel gene family. NF-κB subunit proteins (p50, p65, *etc*.) exist as dimers and re-

side in the cytoplasm, where they are sequestered as a complex with IκB, a family of inhibitory proteins that prevent nuclear translocation. Upon stimulation, IκB becomes phosphorylated, causing its dissociation from the NF-κB dimer, followed by ubiquitination and degradation of IκB. The NF-κB transcription factor translocates to the nucleus and can interact with DNA, binding to target sequences and activating transcription. NF-κB regulates the expression of genes encoding inflammatory and immune modulatory proteins, as well as cell survival genes (Baeuerle and Baltimore, 1996; Janssen-Heininger *et al.*, 2000).

Activation of the transcription factor NF-κB has been shown to reduce apoptosis and promote survival in certain cell systems, and plays a key role in inhibition of apoptosis triggered by tumor necrosis factor-α (TNF-α) (Beg and Baltimore, 1996; Cimino *et al.*, 1997; Van Antwerp *et al.*, 1996; Wang *et al.*, 1996). Binding of TNF-α to its receptor results in the recruitment of an adaptor protein, TRAF-2, which in turn causes the activation of NF-κB inducing kinase (NIK). NIK then activates IκB kinases (IKK) α and β. These IKK phosphorylate IκB complexes, thereby targeting IκB for ubiquitination and subsequent degradation through the proteasome pathway.

NK-κB acts as a survival factor that prevents apoptosis in response to cytokines, chemotherapeutic agents, and other agents (Baeuerle *et al.*, 1996; Beg and Baltimore, 1996; Wang *et al.*, 1996; Mayo *et al.*, 1997). Prevention of NF-κB activation by expression of a construct termed the "IκB super repressor" results in enhanced apoptosis in response to oncogenic *ras* (Mayo *et al.*, 1997). Our laboratory has demonstrated activation of NF-κB following exposure of a number of pulmonary cell types to asbestos and oxidants, including H_2O_2, *in vitro* (Janssen *et al.*, 1995, 1997). Moreover, we have shown increases in p65 protein, a subunit of NF-κB, in bronchial epithelial cells after inhalation of either crocidolite or chrysotile asbestos (Janssen *et al.*, 1997). Because asbestos and other oxidants cause cell injury and apoptosis (BeruBe *et al.*, 1996a; Jimenez *et al.*, 1997), NF-κB activation by these agents may inhibit apoptosis and be important in repair following injury. Because genes regulated by NF-κB can also encode chemotactic factors and inflammatory cytokines associated with inflammation (Janssen-Heininger *et al.*, 1999) and the development of asthma and fibrotic lung disease (BeruBe *et al.*, 1996a; Barnes and Adcock, 1998), NF-κB activation may also promote lung injury and disease. Recently, the NF-κB signaling pathway in vascular smooth muscle cells (VSMC) and defects in the regulation of apoptosis have been implicated in the development of atherosclerosis (Tanaka *et al.*, 1995; Rembold, 1996; Bourcier *et al.*, 1997; Davies, 1997).

NF-κB and AP-1 in diabetes, vascular disease, and aging

Activation of the redox-sensitive transcription factors NF-κB and AP-1 are closely linked to the development of a number of diseases (Baeuerle *et al.*, 1994; Sen and Packer, 1996; Barnes and Karin, 1997). NF-κB activation might play a role in the development of complications associated with diabetes (Bierhaus *et al.*, 1997; Morigi *et al.*, 1998) and the pathogenesis of atherosclerosis (Collins, 1993; Bourcier *et al.*, 1997; Abe and Berk, 1998; Kumar *et al.*, 1999). Increased NF-κB binding activity is observed in peripheral blood monocytes isolated from poorly controlled type I diabetic patients (Hofmann *et al.*, 1998), and both AP-1 and NF-κB activation are seen in cardiac tissues of diabetic rats 4 and 24 weeks after the onset of the disease (Nishio *et al.*, 1998). Furthermore, hyperglycemia, accumulation of advanced glycation endproducts (AGE), increased oxidative stress, and oxidant generation in conjunction with decreased antioxidant defense mechanisms can all lead to activation of NF-κB (Schmidt *et al.*, 1995; Sen and Packer, 1996; Bierhaus *et al.*, 1997; Kumar *et al.*, 1999). Gene products induced by NF-κB (*e.g.*, cytokines, inducible nitric oxide synthase, leukocyte adhesion molecules, and tissue factors) are also associated with the development of diabetic complications (Ross, 1993; Abe and Berk, 1998; Alexander, 1998; Griendling and Ushio-Fukai, 1998). Moreover, both NF-κB and AP-1 can contribute to vascular disease by influencing the balance between proliferation and apoptosis of VSMC (Ross, 1993; Johnson *et al.*, 1996; Griendling and Ushio-Fukai, 1998). Apoptosis may facilitate the elimination of normal cells no longer required for the remodeling of tissues as

well as abnormal cells exhibiting neoplastic phenotypes. Because injury or inflammation is an important trigger for the onset of vascular disease, the balance between apoptosis and proliferation during healing as well as aberrations in this process may be important in the progression of the lesion (Isner *et al*., 1995; Johnson *et al*., 1996).

Activation of NF-κB and AP-1 by TNF-α (Beg and Baltimore, 1996; Sen and Packer, 1996; Van Antwerp *et al*., 1996; Wang *et al*., 1996) and their subsequent effects on smooth muscle cell proliferation (Stewart *et al*., 1995; Tanaka *et al*., 1995; Jovinge *et al*., 1997; Mattana *et al*., 1997) have also been described. TNF-α mediates some of its effects through oxidative stress, as it causes increased expression of manganese superoxide dismutase (MnSOD), found in mitochondria (Wong *et al*., 1989) and superoxide production in human fibroblasts *in vitro*. Induction of MnSOD has been shown to be important in cellular resistance to TNF-α, implicating mitochondrial function and concomitant superoxide generation as a key component of TNF-α-mediated tumor cell killing (Wong *et al*., 1989). TNF-α-induced activation of NADPH oxidase, which leads to superoxide production, has also recently been shown in VSMC (De Keulenaer *et al*., 1998). The proliferative effect of TNF-α may also be dependent on c-Jun/AP-1 (Brach *et al*., 1993).

The decline in the immune function of aged individuals and experimental animals correlates with changes in the induction of cytokines, including increased expression of IL-6 which results from the constitutive activation of NF-κB in many cell types present in the spleens of aged mice (Ershler *et al*., 1993; Spencer *et al*., 1997). Helenius *et al*., found an age-related up-regulation of nuclear binding by NF-κB in mouse cardiac muscles (Helenius *et al*., 1996a,b). Vascular disease is also attributed to inflammatory processes (Ross, 1993; Mattana *et al*., 1997), and the increased expression of inflammatory cytokines with advancing age may be related to the high prevalence of atherosclerosis in older people. Selective repression of c-*fos*, one of the components of AP-1, has also been reported in senescent cells *in vitro* (Seshadri and Campisi, 1990), and decreased c-*fos* expression *in vivo* and *in vitro* occurs in cardiac tissue with age (Tsou *et al*., 1996). Others have shown impaired AP-1 activation with age (Whisler *et al*., 1996) leading to decreased interleukin-2 (IL-2) production by human T cells. However, the effect of age in the presence or absence of high glucose on transcription factor activation is not known. We describe below several approaches to measure cell signaling events and transcription factor activation that may be applicable to the elucidation of the pathogenesis signaling processes in diabetes mellitus, atherosclerosis, and aging.

APPROACHES FOR MEASURING CELL SIGNALING PATHWAYS

Molecular and biochemical techniques

A number of molecular techniques are used to examine the activity of specific kinases in signal transduction pathways that lead to AP-1 or NF-κB activation, including kinase activity assays, Western blotting, and electrophoretic mobility shift assays (EMSA). For example, in the MAPK cascade, the activity of ERKs 1 and 2 (ERK1/2) is often measured in kinase activity assays using immunoprecipitates of ERK1/2 from whole-cell extracts and quantitation of the radiolabel, [γ^{32}P]ATP, transferred to an exogenous protein substrate (Jimenez *et al*., 1997; Zanella *et al*., 1999). Antibodies that recognize phosphorylated ERK1/2 are specific for the phosphorylated form of the kinases and do not cross react with the unphosphorylated form or other phosphorylated proteins, thus providing a high degree of specificity for the measurement of both the quantity of protein and its functional state. Western blot analysis using antibodies to phosphorylated ERK1/2 provides a nonradioactive alternative for determining the quantity of "active" ERK1/2 in a cell lysate (Zanella *et al*., 1999).

Analysis of signaling proteins by kinase activity assays or Western blot analyses does not provide evidence that a transcription factor is actually capable of binding DNA, a step critical for the initiation of transcription. To demonstrate DNA binding activity by AP-1 or NK-κB, EMSA is commonly employed. In this assay, consensus oligonucleotides representing AP-1

or NK-κB DNA binding sequences from the promoter region of the genes of interest are radiolabeled in an *in vitro* kinase reaction, purified, and incubated with a few micrograms of extracted nuclear protein to allow formation of specific protein–DNA complexes. These are then resolved in non-denaturing acrylamide gels and detected by autoradiography or phosphorimaging (Heintz *et al.*, 1993; Janssen-Heininger *et al.*, 1999). The unbound oligonucleotide probe migrates rapidly through the gel whereas DNA–protein complexes exhibit a "mobility shift" and migrate more slowly. The specificity of the oligonucleotide probe sequence allows identification of the transcription factor binding to it, but it is also important to establish the subunit composition of the binding complex.

Both AP-1 and NF-κB bind to DNA as homo- or heterodimers comprised of different family members. To identify components of the DNA-binding complex, antibodies recognizing epitopes specific to a single component are incubated in combination with the nuclear extract and the radiolabeled oligonucleotide probe. Binding of an antibody to a component of the DNA-binding complex further slows migration of the complex, causing a "supershift" in the position of the complex when resolved on a nondenaturing acrylamide gel. Using this technique, it is possible to determine, for example, whether the NF-κB present in a nuclear extract is composed of transcriptionally active p50/p65 heterodimers or transcriptionally inactive p50/p50 homodimers, which both exhibit similar DNA-binding activities but are dramatically different in their abilities to initiate transcription (Janssen *et al.*, 1997). Figure 1 shows a representative EMSA and supershift analysis for VSMC grown in high-glucose media or exposed to H_2O_2 or TNF-α. Similarly, the subunit of composition of AP-1 can be identified using specific antibodies to the different components.

The DNA binding activity of a transcription factor is distinct from its ability to activate gene transcription. To assess directly transcriptional activation by AP-1 or NF-κB, two approaches are often employed: (1) inducation of a reporter gene expression (Timblin *et al.*, 1995) and (2) analysis of specific mRNAs encoded by AP-1 or NF-κB-regulated genes (Heintz *et al.*, 1993).

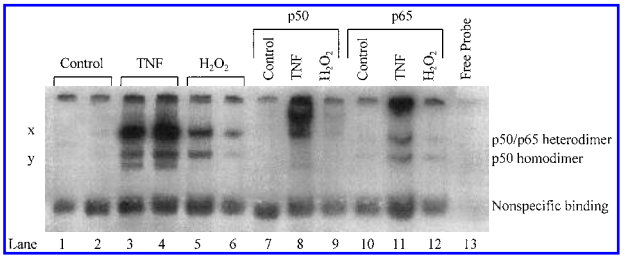

FIG. 1. Example of an EMSA on nuclear extracts from vascular smooth muscle cells. Specific complexes of NF-κB are denoted (x, y). Relative to control (lanes 1–2), TNF-α (lanes 3–4) and H_2O_2 (lanes 5–6) treatments induced NF-κB binding activity (darker bands). Addition of antibodies to p50 and p65 (lanes 7–9 and lanes 10–12, respectively) demonstrate a supershift establishing NF-κB specificity. Lane 13 shows unlabeled oligonucleotide (free probe) used for competition, demonstrating specificity of the binding.

Reporter cell lines that have several copies of an AP-1 or NF-κB DNA response element coupled to the luciferase reporter gene are often used to examine the kinetics of AP-1- or NF-κB-dependent gene transcription in response to extracellular stimuli. In this approach, the amount of luciferase activity (encoded by the reporter gene and transcriptionally regulated by AP-1 or NF-κB, depending on the construct) is a direct measurement of transcription factor activity. The amount of luciferase activity is easily determined in a biochemical assay using whole cell lysates.

Transcriptional activation can also be assessed by examining the steady-state levels of specific mRNAs encoded by genes that are dependent on AP-1 or NF-κB activity. Total RNA prepared from cultured cells or frozen tissues can be analyzed by either Northern blot analysis or ribonuclease protection assays (RPA) for determination of specific mRNA levels. Northern blot analysis is often used to examine the levels of a single mRNA species immobilized on a solid support, such as nitrocellulose membrane, and detected using a specific radiolabeled cDNA probe (Heintz *et al.*, 1993), whereas multiple species of mRNAs are readily detected by RPA (Fig. 2). RPA uses high-specific-activity RNA probes, synthesized from DNA templates, hybridized in solution in excess to target RNA. Following hybridization, free probe and other single-stranded RNAs are digested with a cocktail of ribonucleases (RNases) and the remaining "RNase-protected" probes are purified and resolved on denaturing polyacrylamide gels. In

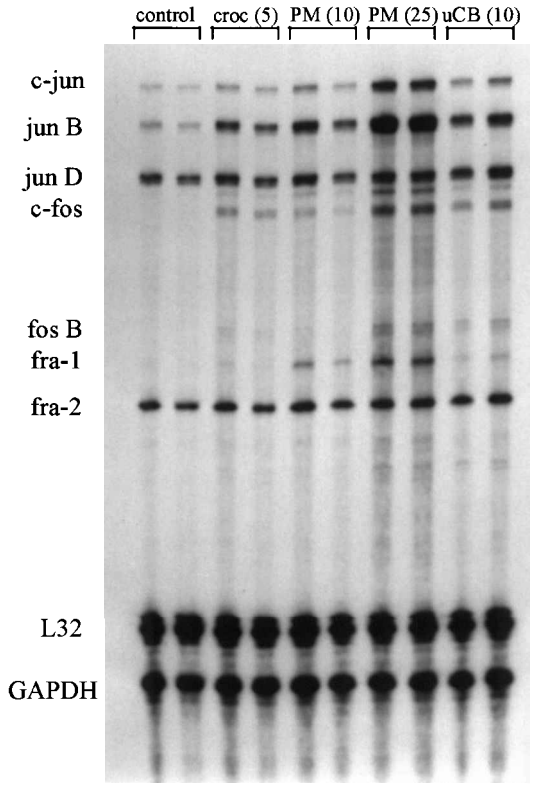

FIG. 2. Example of an autoradiogram from a ribonuclease protein assay (RPA) using a riboprobe template set (Pharmigen, San Diego, CA) for *fos* and *jun* family mRNAs. A murine epithelial cell line (C10) was exposed *in vitro* to 5 $\mu g/cm^2$ crocidolite asbestos (croc), a type of asbestos inducing oxidative stress, 10 or 25 $\mu g/cm^2$ ambient particulate matter (PM), or 10 $\mu g/cm^2$ ultrafine carbon black particles (uCB) and total RNA isolated for RPA. Note that elevated levels of several *fos* and *jun* mRNAs are observed in samples from particle-treated cells compared to unexposed control cultures.

both Northern blot analysis and RPA, the quantity of each mRNA species in the original sample can be determined based on the intensity of the detected band. The intensity is expressed as a ratio to mRNA levels of "housekeeping" genes such as the ribosomal gene, L32, which corrects for heterogeneity in loading and is largely unaffected by various activating agents including oxidative stress.

Cell imaging approaches

The methods described above provide important information on key phosphorylation events as well as whether or not increased protein binding to DNA and transcriptional activation of AP-1- and NF-κB-regulated genes occur in cells and tissues. These molecular events can be correlated with cell responses and detection of proteins comprising signaling cascades using microscopic cell imaging techniques. In addition, identification of the phenotype of cells expressing these proteins, *i.e.*, whether they are apoptotic, proliferative, *etc.*, provides insight into the role of various signaling cascades in governing cell responses. For example, increases in DNA synthesis, which may reflect cell proliferation and/or altered repair processes, are observed in cells after exposure to oxidative stress. Cells in S phase can be identified by incorporation of the DNA analog, 5′-bromo-2′-deoxyuridine (BrdU) (BeruBe *et al.*, 1996b) or with the use of antibodies to proliferating cell nuclear antigen (PCNA) (van Dierendonck *et al.*, 1991). Other complementary immunocytochemical and biochemical techniques are also available to document cell proliferation or apoptosis (Mossman, 1999).

Both immunoperoxidase and multifluorescence techniques can be used to localize phosphorylated and other signaling proteins expressed in cells and tissues (Mossman, 1999; Poynter *et al.*, 1999). Immunoperoxidase methods are generally not as sensitive in detection, but are amenable to light microscopy, whereas multifluorescence approaches allow co-localization of multiple proteins in the same cell using different fluorescent labels. Moreover, the latter can be used in combination with *in situ* hybridization to determine whether AP-1 or NF-κB genes are expressed in cells expressing signaling proteins or signatures of apoptosis or cell proliferation (Davis *et al.*, 1997). Figure 3 shows an example of the use of dual fluorescent labels and confocal scanning laser microscopy to demonstrate expression and translocation of phosphorylated-ERK proteins from the cytoplasm to the nucleus of pulmonary epithelial cells after exposure to 200 μM H_2O_2, a concentration known to induce apoptosis (Buder-Hoffman *et al.*, submitted, and in review). In brief, cells were exposed over a 24-hr time period to H_2O_2. At various time points, coverslips of sham control and H_2O_2-treated cells were examined using a primary antibody detecting phosphorylated-ERK (New England Biolabs, Beverly, MA) and a secondary antibody solu-

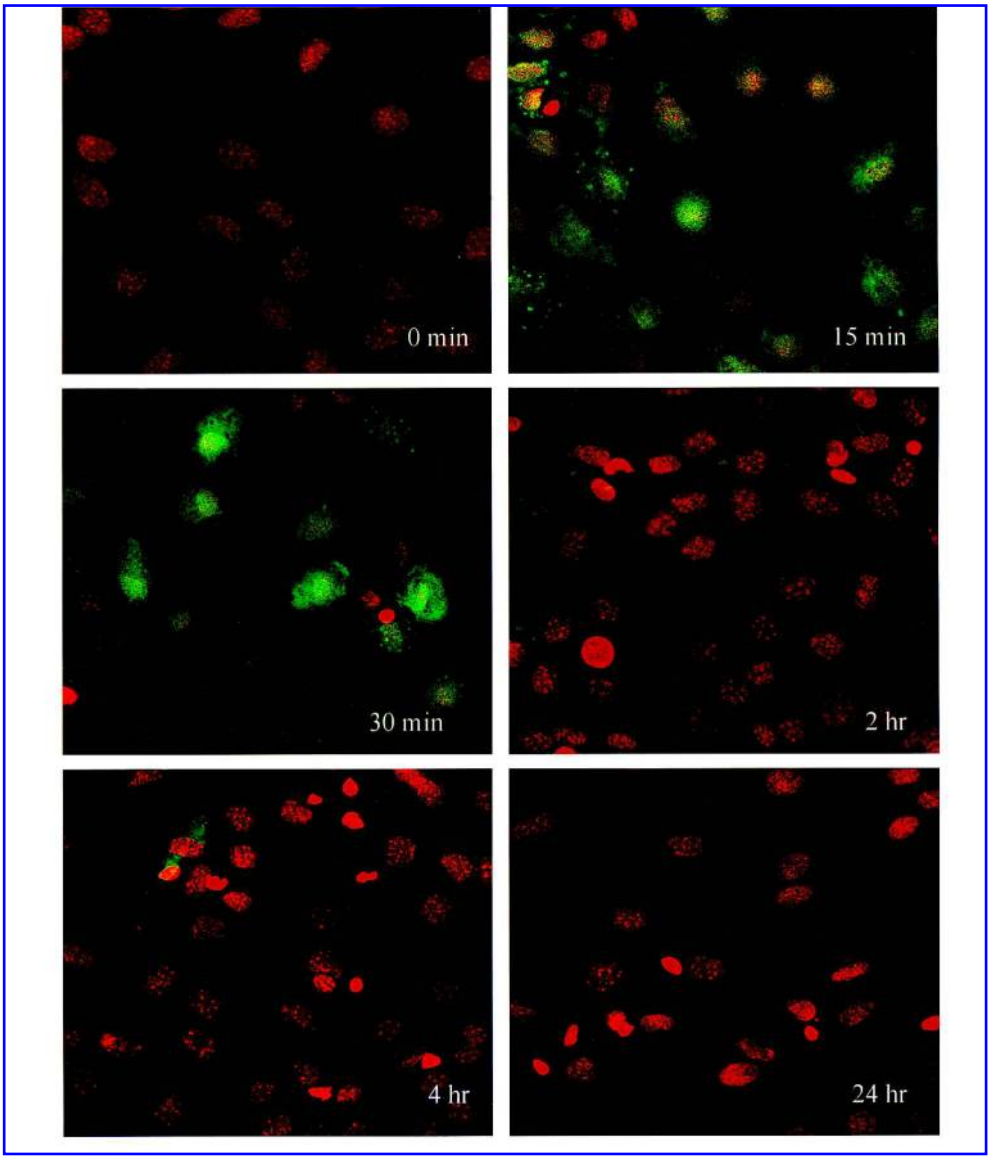

FIG. 3. Localization of phosphorylated-ERK 1/2 proteins (green) in C10 alveolar type II epithelial cells after addition of 200 μM H_2O_2 using confocal scanning laser microscopy. The nuclei are stained with propidium iodide (red). Within 15 min, increased cytoplasmic phosphorylated-ERK proteins and nuclear translocation (yellow) occurs. By 2 hr, these changes are gone, and by 24 hr, some apoptotic cells with shrunken or fragmented nuclei are observed.

tion conjugated to a fluorescent label (Alexa 488). Cells were subsequently incubated in a propidium iodide (PI) solution to localize nuclear DNA, which appears red when collected in the fluorescence mode of a confocal scanning laser microscope whereas the phosphorylated-ERK proteins appear green (Fig. 3). Separate confocal images are then collected in the fluorescence modes followed by computerized merging of the images which appears yellow, thus indicating co-localization. Data thus provide a morphologic assessment of cytoplasmic versus nuclear localization, and can be correlated with results of EMSA studies. The time frame, patterns, and duration of ERK phosphorylation may be critical in signaling and subsequent cellular responses elicited by ERK-activating stimuli. For example, the data in Fig. 3 show that rapid and transient phosphorylated-ERK nuclear translocation occurs in cells exposed to H_2O_2, an agent inducing rapid apoptosis. In constrast, exposure to the oxidant

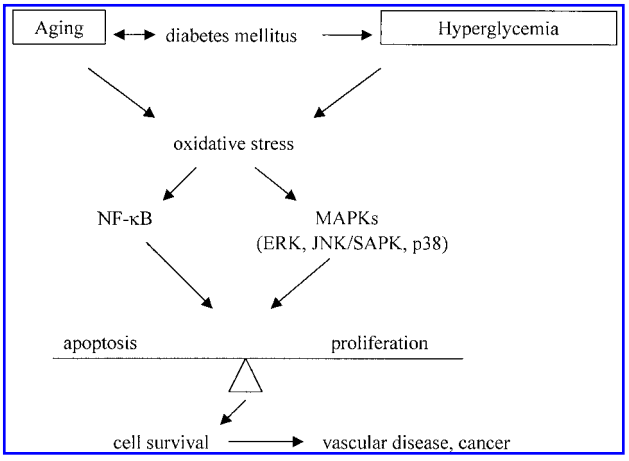

FIG. 4. Schema of interrelationships among oxidative stress, aging, and signal transduction pathways involved in cell proliferation, apoptosis, and survival.

stress asbestos causes a protracted ERK phosphorylation with significant increases in protein expression by Western blot analysis and nuclear translocation occurring after 4 hr (Buder-Hoffman *et al.*, submitted, and in review). These events are followed by apoptosis at 24 h and proliferation occurring at 72 h.

A promising new tool that can be used to combine cell imaging methods and flow cytometry is the laser scanning cytometer (LSC) (Deptala *et al.*, 1998; Mossman, 1999). Using this apparatus, cells on the same slide can be relocated using computer-controlled methodology and restained with a number of fluorophores and dyes including PI to document cell cycle distribution. Representative cells also can be mapped for localization of proteins in plasma membrane, cytoplasmic, and nuclear compartments using partitioning approaches. This technique has been used to map the nuclear translocation of p65 protein in cells after exposure to agents stimulating NF-κB activity (Deptala *et al.*, 1998).

Another approach afforded by LSC is the use of green fluorescent protein (GFP) in co-transfection studies to introduce modified signaling proteins or dominant-negative constructs into cells (Palmer *et al.*, in preparation). For example, cells can be transiently co-transfected with GFP and constructs of interest, and populations of untransfected and transfected (GFP-positive) cells assessed individually after PI staining using fluorescence detectors to determine whether cell cycle kinetics, *i.e.*, proportions of cells in S phase and/or apoptotic fractions (sub-G_0/G_1), are altered. This methodology will be a valuable tool to determine whether stimulation of certain signaling proteins by oxidative stresses or other insults are causally related to cell proliferation and apoptosis in a number of cell types.

SUMMARY

As a consequence of the recognition that oxidative stress may be an adverse factor in the aging process and in the pathogenesis of a large number of chronic diseases, there is increasing interest in techniques to study redox-sensitive pathways and to relate them to phenotypic consequences. We describe biochemical, molecular, and imaging techniques that may be applicable to investigations in multiple aging paradigms and oxidant-associated diseases, including atherosclerosis and diabetes mellitus. Although oxidants may have deleterious effects, they also play key roles as signal transducers. The pathophysiology of aging may reflect an imbalance in the role of oxidants as mediators of disease and as important components of signal transduction pathways (Fig. 4). The advent of interventions directed at modulating these pathways will be enhanced by the

understanding that these tissue-specific assays will afford.

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ABBREVIATIONS

AGE, Advanced glycation end products; AP-1; activator protein-1; ATP, adenosine triphosphate; BrdU, 5′-bromo-2′-deoxyuridine; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal regulated kinases; GFP, green fluorescent protein; H_2O_2, hydrogen peroxide; IKK, IκB kinases; IL-2, interleukin-2; JNK/SAPK, c-Jun N-terminal/ stress-activated protein kinases; LSC, laser scanning cytometer; MAPK, mitogen-activated protein kinase; Mn-SOD, manganese superoxide dismutase; NF-κB, nuclear factor-kapp B; NIK, NF-κB-inducing kinase; $\dot{O}_2^-$, superoxide; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; RNase, ribonuclease; ROS, reactive oxygen species; RPA, ribonuclease protein assays; TNF-α, tumor necrosis factor-α; VSMC, vascular smooth muscle cells.

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