CMLS, Cell. Mol. Life Sci. 57 (2000) 1287–1305
1420-682X/00/091287-19 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

Signaling by reactive oxygen species in the nervous system

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Received 6 March 2000; received after revision 10 April 2000; accepted 13 April 2000

Abstract. Free radicals and reactive oxygen species (ROS) are involved in a variety of different cellular processes ranging from apoptosis and necrosis to cell proliferation and carcinogenesis. Cells contain multiple sites for ROS production and a few mechanisms for their degradation. Which of these sites is activated by a given stimulus may play a role in dictating the subsequent downstream effects of the ROS generated on cellular function. Even when the ultimate outcome is similar, such as when ROS production leads to cell death, the specific cellular changes can be quite different depending on the initial stimulus and the type of cell involved. These data, along with the evidence that ROS can modify a number of intracellular signaling pathways including protein phosphatases, protein kinases and transcription factors, suggest that the majority of the effects of ROS on cells are through their actions on signaling pathways rather than via nonspecific damage of intracellular macromolecules.

Key words. ROS; apoptosis; necrosis; glutathione; H_2O_2; transcription factors; tyrosine phosphatases; protein kinases.

Introduction

Although the potential toxicity of molecular oxygen (O_2) was recognized in the 18th century by Lavoisier, Priestley and Scheele, it was not until 1954 that Rebeca Gerschman did the compelling experiments to show that O_2 toxicity was mediated by oxidizing free radicals [1]. Nineteen years later Babior demonstrated that the superoxide radical ($O_2^{\cdot -}$) is produced by leukocytes as a defense mechanism against bacteria [2], and the scientific community began to appreciate the possibility that free radicals and reactive oxygen species (ROS) may play significant roles in cell physiology. Today the experimental literature is replete with data showing that this group of highly reactive molecules is involved in everything from apoptosis and necrosis to carcinogenicity and aging. Clearly, a single review can cover but a small fraction of this enormous and rapidly expanding area of scientific endeavor. Since the authors' expertise is in the areas of neurobiology and growth factors, this review will focus upon the role of ROS in stress responses and cell death within the central nervous system (CNS). We will not discuss nitric oxide nor will we discuss in detail the enormous literature on p53 and nuclear factor κB (NF-κB) in apoptosis (see for example [3, 4]). Initially, a primer on free-radical chemistry will be presented, followed by a discussion of potential sources of free radicals and ROS in mammalian cells. This will be followed by an outline of our current mechanistic knowledge of the physiological roles of ROS and free radicals in cell death and stress. An

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attempt is made to draw attention to pathways that probably play a central role in most forms of cell death, but have not received the attention they deserve. Finally, we will use the term programmed cell death in reference to all forms of death which require active cellular participation (i.e. gene transcription and/or enzyme activation) in order for the cells to die in response to a stressor. A justification for this use of the term will be presented after the discussion on apoptosis and necrosis.

Reactive oxygen species and free radicals

Oxidation is the loss of electrons by an atom or molecule, and therefore an oxidizing agent is good at absorbing electrons from the molecule or atom it oxidizes. Free radicals, which contain one or more unpaired electrons, and ROS, which are radical derivatives of molecular oxygen as well as hydrogen peroxide (H_2O_2), are therefore good oxidizing agents. Biologically relevant ROS include the superoxide radical ($O_2^{\cdot -}$), hydroperoxyl radicals ($HO_2^{\cdot}$), H_2O_2 and the hydroxyl radical (·OH). Of these, the hydroxyl radical is the most damaging, for it reacts with many macromolecules with a rate constant approaching diffusion-controlled. In contrast, $O_2^{\cdot -}$ reacts very slowly, and some consider it to be a sink for radical electrons. Additional members of this family are derived from the interaction of carbon- and nitrogen-based radicals with O_2. These include organic peroxides, alkoxyl (RO·) and peroxyl (RO_2·) radicals, and peroxynitrite ($ONOO^-$) and nitric oxide (·NO). The extensive reactions which occur between this family of molecules and the components of cells has been thoroughly reviewed [5, 6] and will not be discussed here except to emphasize a point of discussion. All free radicals and many ROS derivatives can, however, cause extensive damage to macromolecules through the formation of adducts, the destruction of unsaturated C–C bonds, and the oxidation of disulfides. These unique properties have allowed ROS to evolve into functional roles in cell death and as exquisitely sensitive signaling molecules within cells.

What are the sources of ROS?

There are two ubiquitous sources of ROS in eukaryotic cells. The first source of free radicals is from the effect of ionizing radiation on water, which generates a variety of initial products, many of which can go on to form additional reactive molecules.

$$H_2O \rightarrow \rightarrow \rightarrow e_{aq}^-,\ \cdot OH, \cdot H, H_3O^+, H_2, H_2O_2$$

Since both cosmic and terrestrial background radiation contribute to this source of ROS, it is clear why organisms have evolved elaborate defense systems to deal with the problem of ROS production (to be discussed later). The second source results from the development of aerobic metabolism about 1.5 million years ago.

Figure 1 is a schematic representation of the flow of electrons through mitochondria, a process that is known in great detail (for review see [7]). While the goal of the respiratory chain is to use metabolites to produce energy from the reduction O_2 to water, this series of reactions is inefficient, resulting in the production of ROS from the interaction of molecular oxygen with free electrons. It was initially shown that isolated mitochon-

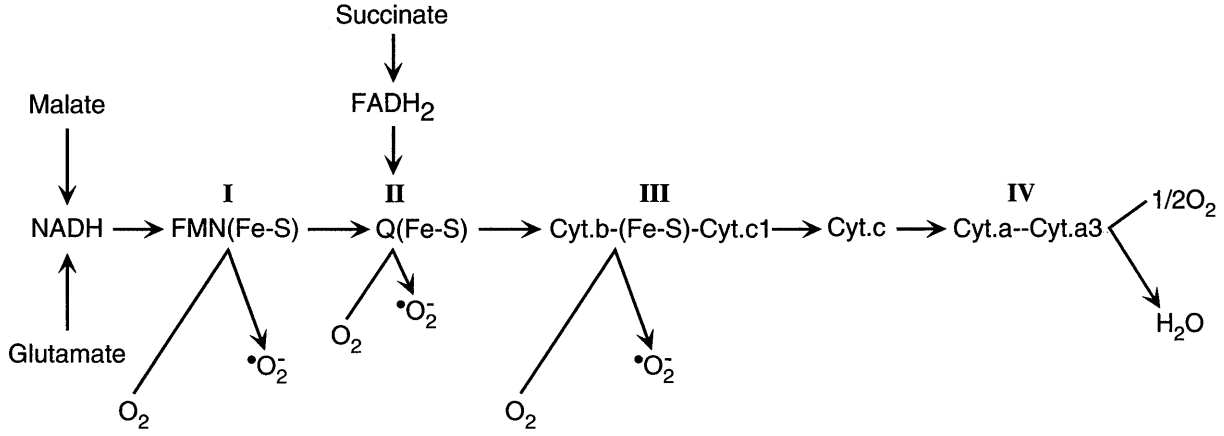

Figure 1. Production of superoxide by the mitochondrial electron transport chain. Although it is generally agreed that mitochondria produce significant amounts of superoxide under physiological conditions, the relative contribution of each complex is controversial. However, the consensus is that the majority of the superoxide comes from complexes I and III, as indicated in the figure.

dria produce H_2O_2 (derived from $O_2^{\cdot -}$) from both nicotinamide adenine dinucleotide (NAD)- and flavin adenine dinucleotide (FAD)-linked substrates [8]. Although still controversial, it is currently believed that the major sources of mitochondrial H_2O_2 production are from the ubiquinone sites in complexes I [NADH (the reduced form of NAD)-ubiquinone reductase] and III (ubiquinal-cytochrome C reductase), with a less substantial contribution from complex II [8–11]. There is no evidence that ROS are generated during the final step of O_2 reduction to H_2O via cytochrome oxidase. The 'leak' of electrons from the upstream components of the chain to O_2 results in the formation of $O_2^{\cdot -}$. $O_2^{\cdot -}$ is then dismutated to H_2O_2 by mitochondrial superoxide dismutase (SOD), and H_2O_2 is converted in the presence of iron to the highly reactive hydroxyl radical, a reaction described by Fenton in 1894.

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + {\cdot}OH$$

When electron transfer through mitochondria is reversed by the presence of excess ATP and succinate, there is a dramatic increase in $O_2^{\cdot -}$ production [11], suggesting that a high ADP/ATP ratio due to energy utilization may minimize ROS production by mitochondria. Such a condition may contribute to the observation that calorie restriction promotes longevity in some animals.

Although the physiological source of ROS in most cell death pathways appears to be mitochondria, many other sources of ROS have been identified in the CNS. Indeed, several are more potent than mitochondria. The best studied of these is the NADPH (reduced form of NAD phosphate) oxidase of macrophages and CNS microglia (for review see [12]). Although the exquisitely regulated NADPH oxidase complex was initially thought to be confined exclusively to macrophages and microglia, NADPH oxidases have been implicated in growth factor signaling in a variety of cell types (see for example [13–15]), including nerve cells [16]. The latter manuscript showed that the five required subunits of NADPH oxidase are present in sympathetic neurons and that the activity of this complex is required for the programmed cell death associated with NGF withdrawal. These data therefore expand the potential number of sources for ROS signaling and perhaps toxicity within the nervous system. Other, less cell type specific, sources of ROS include the large numbers of oxidases associated with peroxisomes, endoplasmic reticulum and cytoplasm. These include lipoxygenases and cyclooxygenases, cytochrome P450 oxidases, monoamine oxidases, and nitric oxide synthase. In addition, there are nonenzymatic sources such as catecholamine autoxidation. These abundant sources of ROS, as well as the brain's extraordinarily high rate of metabolism due to the requirement for pumping ions associated with synaptic communication (the brain uses about 20% of the body's total O_2 intake despite the fact that it has only about 2% of the total body weight. Therefore the respiration rate is 10 times higher than that of average tissue) has forced CNS cells to evolve an elaborate machinery for protection from ROS. In addition, the high levels of the transition metals iron and copper in certain neuronal cells support Fenton chemistry to generate ·OH, and the many unsaturated lipids in nerve cell membranes are susceptible to lipid peroxidation.

Defense systems

Due to both the background generation of ROS from ambient radiation and the endogenous generation of cellular ROS outlined above, cells have evolved an elaborate series of mechanisms to minimize ROS presence and reduce damage if it occurs. Figure 2 outlines the pathways involved in ROS generation and control. Within the CNS, it is likely that the major enzymatic system for maintaining low levels of the more dangerous ROS is glutathione peroxidase (GPx) and the enzymes associated with maintaining high levels of intracellular reduced glutathione (GSH). GSH is a tripeptide (γ-glu-cys-gly) characterized by De Rey-Pailhade over 100 years ago [17]. Since the glutathione thiol has a low oxidation potential and therefore reducing power, it is a potent antioxidant. Its intracellular concentration is between 2 and 10 mM, of which 99% is normally in the reduced state [99% GSH vs. 1% glutathione disulfide (GSSG)]. Both GPx and catalase can reduce peroxides (H_2O_2 or ROOH) to water or organic alcohols (ROH), but in the nervous system the major player seems to be GPx, for it is both more abundant, particularly in mitochondria, and has a higher turnover rate than catalase. Superoxide dismutase (SOD) is a double-edged sword, for it converts the relatively innocuous $O_2^{\cdot -}$ to H_2O_2, which is readily degraded to the very reactive ·OH via the Fenton reaction. Since Fenton chemistry requires multivalent iron or copper to mediate electron transfer, sequestration of these metals by molecules such as ferritin (which retains about 4500 iron ions per molecule of protein) can also serve as a defense mechanism against free radical damage and therefore should be considered an antioxidant. The antioxidant vitamins E and C are also important in the CNS [18]. Other enzymes such as heme oxygenase and quinone reductase can mitigate radical formation, and a number of cysteine-rich proteins such as metallothionein, thioredoxin (Trx) and redox factor 1 can behave like GSH to maintain the redox status of cells and minimize oxidative stress.

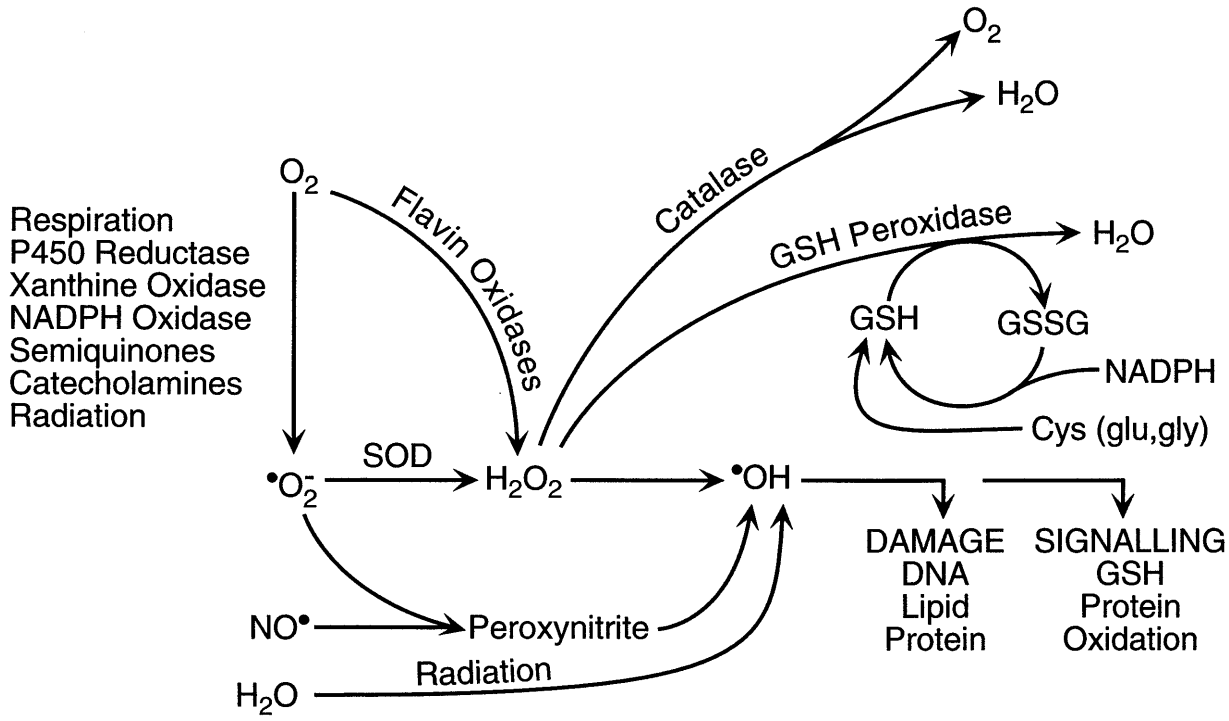

Figure 2. Sources of ROS and mechanisms for their removal from cells. SOD, superoxide dismutase; GSH, glutathione.

What are 'oxidative stress' and 'redox status'?

It has generally been assumed that oxidative stress and an imbalanced redox homeostasis are functionally the same with respect to generation and consequences—they occur when there is either an overproduction of ROS or a deficiency in the sum total of the antioxidant systems. This may not, however, be the case, for it is likely that under some forms of stress there is a deficiency in total reducing potential (e.g. low GSH, low Trx) in the absence of significant ROS production, and vice versa. Although ROS can lead to the oxidation of protein thiols, it is also likely that thiol-based redox molecules such as GSH and Trx can act directly as signaling molecules in the absence of excessive ROS. For example, when there is an excessive accumulation of GSSG, it can inactivate a number of enzymes and other proteins (e.g. the transcription factor AP-1, see below) by forming mixed disulfides.

$$\text{active } E-SH + GSSG \rightarrow \text{inactive } E-S-SG + GSH$$

Conversely, ROS and other radicals may serve as 'second-message' signals independently of the overall status of thiol redox state, albeit GSH may be lowered by the necessity of detoxifying ROS. Other unique ROS signaling pathways may involve tyrosine nitration [6]. Even within the ROS family, different sensing molecules have been identified in bacteria for H_2O_2 (OxyR) and $O_2^{\bullet -}$ or NO (SoxR) [19]. It can therefore not be assumed that delivering prooxidant stress by different ways either experimentally (e.g. organic vs. inorganic peroxides) or pathologically (ischemia vs. Parkinson disease) will produce the same set of physiological responses. Indeed, the major function of ROS and redox status in the cell is likely to be signaling rather than overt killing.

Bcl-2

Bcl-2 is one of the first proteins shown to regulate apoptosis. It is localized to the outer membranes of mitochondria and nuclei, and forms heterodimers with a family of proteins which contain a unique helical domain called BH3. Bcl-2 and its binding partners may alter ROS production and are clearly involved in some examples of programmed cell death. It is, in fact, frequently assumed that if cell death is inhibited by the overexpression of Bcl-2, then the cells die by a classical apoptotic mechanism. However, since the overexpression of Bcl-2 in cell lines or transgenic animals is often 10-fold or more higher than would ever be found in a normal cell, is the extrapolation from Bcl-2 inhibition to

a specific cell death mechanism valid? New data on the mechanism of Bcl-2 protection make it unlikely that the inhibition of cell death by Bcl-2 is sufficient to warrant the conclusion that death is via classical apoptosis. By expressing Bcl-2 in Bcl-2-negative lymphoma cells, it was shown that Bcl-2 causes about a twofold increase in basal mitochondrial H_2O_2 production [20]. In fact, when Bcl-2 is expressed in bacteria, it acts as a prooxidant [21]. When apoptosis is induced in the non-Bcl-2-expressing cells by C6-ceramide or by tumor necrosis factor (TNF-α), there is an increase in H_2O_2 accumulation. This increase does not occur in the Bcl-2-expressing cells. The mechanism whereby Bcl-2 increases H_2O_2 is probably by increasing the activity of SOD [21, 22]. The selection of stress-resistant cells by growth in the presence of agents which cause cells to produce ROS, such as amyloid or glutamate, also selects for populations with elevated catalase, GPx and other antioxidant activities [23, 24]. Direct selection by growth in the presence of peroxides produces a similar phenotype. Thus, if there is a positive (linear) relationship between the amount of Bcl-2 expression and the induction of ROS defenses, then any process involving a ROS intermediate, be it necrosis or apoptosis, would be blocked. It is likely that Bcl-2 overexpression increases endogenous oxidative stress defense mechanisms which then allow the cells to degrade the ROS produced by TNF-α or ceramide, preventing ROS accumulation. Therefore, the elevated expression of Bcl-2 will produce a more ROS-resistant cell, and one resistant to many forms of oxidative stress [25].

Apoptosis or necrosis?

The initial and defining distinction between necrosis and apoptosis was made on the basis of the ultrastructural characteristics of the dying cell and the observation that cells with an apoptotic ultrastructure tended to be phagocytosed. Apoptosis was defined as 'a mechanism of controlled cell deletion' and as 'an active, inherently programmed phenomenon' [26]. It was identified in tissues by cell shrinkage, fragmentation and chromatin condensation: dying cells are phagocytosed and there is little inflammation. In contrast necrosis, a term for cell death which has been around for over a century, is thought to be an 'irreversible disturbance of cellular homeostatic mechanisms' and is morphologically recognized by cell swelling and mitochondrial destruction. In 1980 Wyllie et al. [27] put together the ultrastructural data of Kerr with the observations from several laboratories that the irradiation of lymphocytes caused the 'laddering' of genomic DNA, into a coherent package of biochemical and morphological criteria for apoptosis. This paper initiated the current wave of interest in the subject. Once apoptosis was studied in additional cell culture systems, a number of other criteria, such as the requirement for de novo protein synthesis, entered the literature and the data on caspases were transferred from *Caenorhabditis elegans* to mammalian cells.

Although it was initially assumed that apoptosis and necrosis were unrelated events, over the last few years exceptions have been found for essentially all of the criteria which initially defined apoptosis, and it now appears to us that there is, in fact, no fundamental distinction between the two processes. Everyone would agree that for apoptosis to occur the cell must somehow participate actively in its own demise (this was indeed part of Kerr's definition), yet this was erroneously assumed not to be the case with necrosis. For example, the depletion of intracellular GSH by buthionine sulfoximine (BSO), a GSH-synthetase inhibitor, causes cell death which appears by all earlier criteria to be necrotic [28]. More recently, however, it has been shown that nerve cell death initiated by GSH depletion is, in fact, a form of programmed cell death which requires macromolecular synthesis, caspase activation, ROS production, and Ca^{++} mobilization [29, 30]. There is, however, no DNA laddering, and the morphology of the dying cells appears more similar to what was initially defined as necrosis [28, 30]. Since both apoptosis and necrosis require a ROS intermediate, it seems to be a mistake to make a global distinction between necrosis and apoptosis at all—they are the ends of a continuum. Indeed, even the initial morphological criteria used to distinguish apoptosis from necrosis are somewhat dubious, and these clearly cannot be applied to cell culture work. For example, the lack of inflammation in apoptosis relative to necrosis may simply reflect the number of dying cells, for apoptotic death usually occurs sparingly within populations. In contrast, classic necrotic cell death is frequently associated with pathological events and usually occurs en masse, resulting in a much greater release of cytokines and other proinflammatory molecules. In fact, when there is extensive localized apoptosis, such as occurs in the leg bud of the chick embryo, there is a very large inflammatory response (for an innovative review of this and other issues being discussed here see [31]). The other defining aspect of apoptosis is cell fragmentation associated with cell shrinkage. However, this combination of phenomena is only seen in animal tissues in vivo, and the cell fragments (apoptotic bodies) are only seen inside other cells. Therefore, the tendency of cells toward shrinking (apoptosis) or swelling (necrosis) could simply reflect the inherent ability of the surrounding cells to phagocytose the cytoplasmic protrusions which occur on the surface of all dying cells. In fact, some cells do swell during apoptosis [31]. It seems that the driving force to

categorize behaviors into extremes is based upon two socioeconomic issues rather than upon science. First, apoptosis is clearly a much flashier way to die than what has classically been termed necrosis, and papers on apoptosis are more likely to be published in high-profile journals. Second, companies promote kits which claim to identify apoptotic cells, even though in actuality these kits do not make the distinction between any classes of dying cells. For example, TUNEL labeling of DNA identifies cells with all kinds of fragmented DNA, but also viable cells undergoing extensive DNA repair or in the process of division. Conversely, BUdR, which is though to label dividing cells, can also label dying cells partaking in DNA repair. Therefore, unless a cell is killed outright by an environmental event or critical aspects of its metabolism are directly inhibited, it cannot be assumed that any type of cell death occurs by a passive necrotic mechanism.

Finally, a few sentences about what is meant by the term programmed cell death. This term was initially used in the context of subpopulations of cells dying at a certain time point during embryonic development. For example, during the development of the mammalian nervous system, large populations of nerve cells die by classical apoptosis at the exact same time and place in each individual. Because of the reproducibility of this phenomenon, it was assumed that the cell death was genetically programmed and not externally driven. We now know, however, that this type of cell death in the nervous system is determined by competition for target tissues and trophic factors and not by any inherent death program within the cells. Therefore, the term programmed cell death as it has been used historically is mechanistically incorrect in many, if not most, cell deaths which occur during development. It seems to us that the term should now be used in the broader sense of any cell death mechanism which requires active cellular participation in terms of macromolecular synthesis or enzyme activation.

Excitotoxicity

Although glutamate is the most abundant neurotransmitter, its extracellular concentration must be maintained below 1 μM, for it is toxic above this level (see for example [32]). The maintenance of low extracellular glutamate is accomplished by five isoforms of sodium-dependent glutamate transporters expressed on neurons and astrocytes (for review see [33]). Glutamate uptake by astrocytes is inhibited by concentrations of H_2O_2 which are probably similar to those generated by CNS pathologies such as stroke, ischemia and trauma. A common target of ROS is the oxidation of sulfhydryl groups, and it is thought that this is the mechanism which leads to the inactivation of the glutamate transporters under conditions of oxidative stress [33].

As with apoptosis, cell death initiated by the activation of ionotropic glutamate receptors, termed excitotoxicity [34], seems to be mediated by mitochondria and ROS. The precise mechanisms of cell death are, however, unclear (see for example [35]). Ca^{++} influx through activated *N*-methyl-D-aspartate (NMDA) receptors (or D,L-amino-3-hydroxy-5-methyl-4-isoazolepropionate [AMPA]/kainate receptors [36]) initiates the cell death pathway, for both NMDA receptor activation and extracellular Ca^{++} are required for the rapid increase in intracellular Ca^{++} [37, 38]. Excess intracellular Ca^{++} uncouples electron transfer from ATP synthesis, and results in the generation of ROS, a process which is autocatalytically augmented by ROS [39]. Ca^{++} also causes the mitochondria to become depolarized, a process which is inhibited by the application of cyclosporin A, which plugs the mitochondrial transition pore [40]. While these results show that the mitochondria and presumably mitochondrially generated ROS are required for excitotoxic cell death, they do not establish a formal role for either in the cell death pathway. It is not known, for example, whether the ROS and/or Ca^{++} are sufficient to lyse the cells, or if additional steps are required before the cells die. Finally, NMDA, but not kainate and AMPA receptors, are also directly sensitive to redox changes, but in the opposite manner of glutamate transporters. Oxidation results in a decrease in Ca^{++} flux through activated NMDA receptors, which may be an evolutionarily derived protective mechanism since Ca^{++} flux through NMDA channels is thought to initiate the excitotoxicity cascade.

Oxidative glutamate toxicity: a necrotic form of apoptosis?

Cell death has been studied in dozens of clonal cell lines, primary cultures and animals using an equally large number of reagents and conditions to initiate death. The outcomes of these experiments are usually catalogued as either apoptosis or necrosis based upon a very limited number of assays. Given that there are exceptions to all criteria for apoptosis, we have argued above that it is a mistake to pigeonhole the death of a cell into one category or another. It is, however, important to understand the underlying mechanisms which lead to cell death. Similarly, most reviews lead the casual reader to believe that apoptosis takes place by the same series events, involving the same cast of molecules, in all cell types. This conclusion is certainly not valid, and in fact, even the same triggering reagent can lead to a different series of events depending upon the concentration and exposure time. To emphasize the

points that death which looks morphologically like necrosis may not be, and that the cast of molecules involved in death can be quite different from the more classical Bcl2/Bax paradigm, we will devote a few paragraphs to a unique form of nerve death in primary CNS neurons and the HT22 hippocampal cell line which could be classified as either necrosis or apoptosis, depending upon the assays employed in its definition. However, it is clearly a form of programmed cell death when studied in detail.

HT22 cells were derived from the mouse hippocampus by SV40 transformation and are probably phenotypically most similar to neuronal precursor cells. They lack ionotropic glutamate receptors, but are killed by exogenous glutamic acid in the millimolar range. The initiating event in this form of glutamate-mediated cell death is the blockade of cystine uptake into the cell via the inhibition of the glutamate/cystine antiporter by exogenous glutamate [41]. This antiporter, which has recently been cloned [42], normally transports the oxidized form of the essential amino acid cysteine down its concentration gradient into cells, coupled with the export of intracellular glutamate. High extracellular glutamate blocks this process, depriving the cell of cystine. Inside the cell, cystine is reduced to cysteine, which is a substrate for glutathione synthesis. In the absence of GSH, cells become oxidatively 'stressed', produce large amounts of ROS, and die—a process termed oxidative glutamate toxicity [43]. The result of the insult could be classified as either necrosis or apoptosis, depending upon the assay. If this type of cell death occurred during CNS development or following CNS trauma and was assayed exclusively by ultrastructural methods, it would certainly be classified as necrosis by the standard morphological criteria (see for example [28]). By biochemical criteria, however, it closely resembles apoptosis, although it lacks the involvement of the Bcl2/Bax pathway (unpublished).

When primary CNS neurons which do not express ionotropic glutamate receptors or the HT22 hippocampal nerve cell line are exposed to millimolar glutamate concentrations, which are readily attainable within the damaged nervous system [44, 45], the cells die within 15 h. By 10 h, the endoplasmic reticulum and Golgi are swollen and are associated with large cytoplasmic vacuoles. Similarly, the mitochondria are swollen and show a loss of cristae. In contrast, the nuclear morphology remains normal, with no evidence of chromatin condensation, a major defining characteristic of apoptosis [30], (see also [28, 46]).

In addition to morphological studies, the glutamate-induced death of these cells has been studied at the biochemical level. Figure 3 outlines the known sequence of events which occurs in the presence of exogenous glutamate in HT22 cells; essentially all of these steps are also required for the death of mouse and rat cortical neurons by oxidative glutamate toxicity. Following exposure of the cells to glutamate, there is a rapid decline in the intracellular antioxidant GSH. If GSH reaches levels below 20% of normal for more than a few hours, the cells initiate a cell death program [47]. If glutamate is removed after shorter periods of GSH depletion, the cells recover. Therefore the nerve cells have a developed mechanism which allows them to be severely stressed (via depletion of GSH) and still survive for short periods of time. Associated with the initial trigger of GSH depletion, at least four other events must occur more or less simultaneously for the initiation of further downstream events which lead to cell death: (i) new messenger RNA (mRNA) and protein synthesis [29, 30]; (ii) the activation of one or more caspases [29, 30]; (iii) the activation of 12-lipoxygenase (12-LOX) [47]; (iv) the influx and metabolism of monoamines [48]. The requirement for protein synthesis and caspase activation occurs at the same time as GSH is being depleted [29], suggesting that the initiation of a decline in GSH levels may be a trigger for the activation of caspase and gene transcription. Little is known about this coupling mechanism at present. The coupling between 12-LOX activation and endogenous GSH depletion is, however, better understood. LOXs are dioxygenases which incorporate molecular oxygen into polyunsaturated fatty acids; 12-LOX, defined by the position of oxygen insertion in the fatty acid, is the predominant CNS form of the enzyme. The major product of 12-LOX in the brain is 12-hydroxyeicosatetraenoic acid (12-HETE). HETE's may play major roles in the modulation of synaptic transmission, and they also activate soluble guanylate cyclase (sGC) via a NO-independent pathway. Li et al. [47] showed that in cortical neurons and HT22 cells the depletion of GSH directly causes the translocation of 12-LOX to the membrane and activates 12-LOX enzymatic activity; inhibitors of 12-LOX block cell death. It was also shown in cell lysates that high concentrations of GSH inhibit 12-LOX activity. The products of 12-LOX activation have at least two functions; the activation of sGC to produce cyclic GMP (cGMP), which in turn activates a channel required for Ca^{++} influx late in the cell death pathway, and a LOX metabolite is required for the very large increase in the production of ROS which occurs around 6 h after the addition of glutamate to cells (fig. 3).

During the first 5 h after the addition of glutamate, there is about a 10-fold linear increase in ROS, which is followed by an exponential increase of over 200-fold relative to baseline [29]. While the initial linear increase in ROS is contemporaneous with the decline in GSH, the large increase only occurs after GSH is depleted below 20% of controls. In addition, the large increase in ROS production requires new mRNA and protein syn-

thesis, caspase activation, 12-LOX activation and an influx of monoamines [29, 30, 47, 48] since the inhibition of any one of these events blocks ROS accumulation and subsequent cell death. The massive increase in ROS is derived from mitochondria, for it can be directly inhibited by FCCP, an uncoupler of oxidative phosphorylation, and the complex III inhibitor antimycin A [29]. ROS production is also inhibited by the NADPH oxidase inhibitor diphenylene iodonium (DPI) and by concentrations of the monoamine oxidase inhibitor clorgyline, which are much higher than required for the inhibition of classical mitochondrial monoamine oxidase [48]. Both compounds do, however, react with a variety of flavoproteins, probably including those involved in the mitochondrial electron transport chain. In HT22 cells, exposure to glutamate leads to mitochondrial membrane hyperpolarization [S. Tan, unpublished]. Although mitochondrial membrane depolarization is usually associated with apoptosis, hyperpolarization can result if ADP is limiting, resulting in the inability of ATP synthase to use the electrochemical potential across the membrane. This condition promotes ROS production by mitochondria. Together, these data suggest, but do not rigorously prove, that the source of the massive burst of ROS following glutamate exposure is the mitochondria. Is it, however, these ROS which directly kill the cells, as is argued in the case of necrosis?

It is frequently assumed that in necrosis there is a large burst of ROS, rapid energy depletion or some other catastrophic metabolic event, which directly kills the cell. This is, however, probably not a valid assumption, and with HT22 cells and cortical neurons exposed to oxidative stress, it is clearly not the case. This is because cell death can be inhibited by blocking the necessary biochemical steps downstream of ROS accumulation,

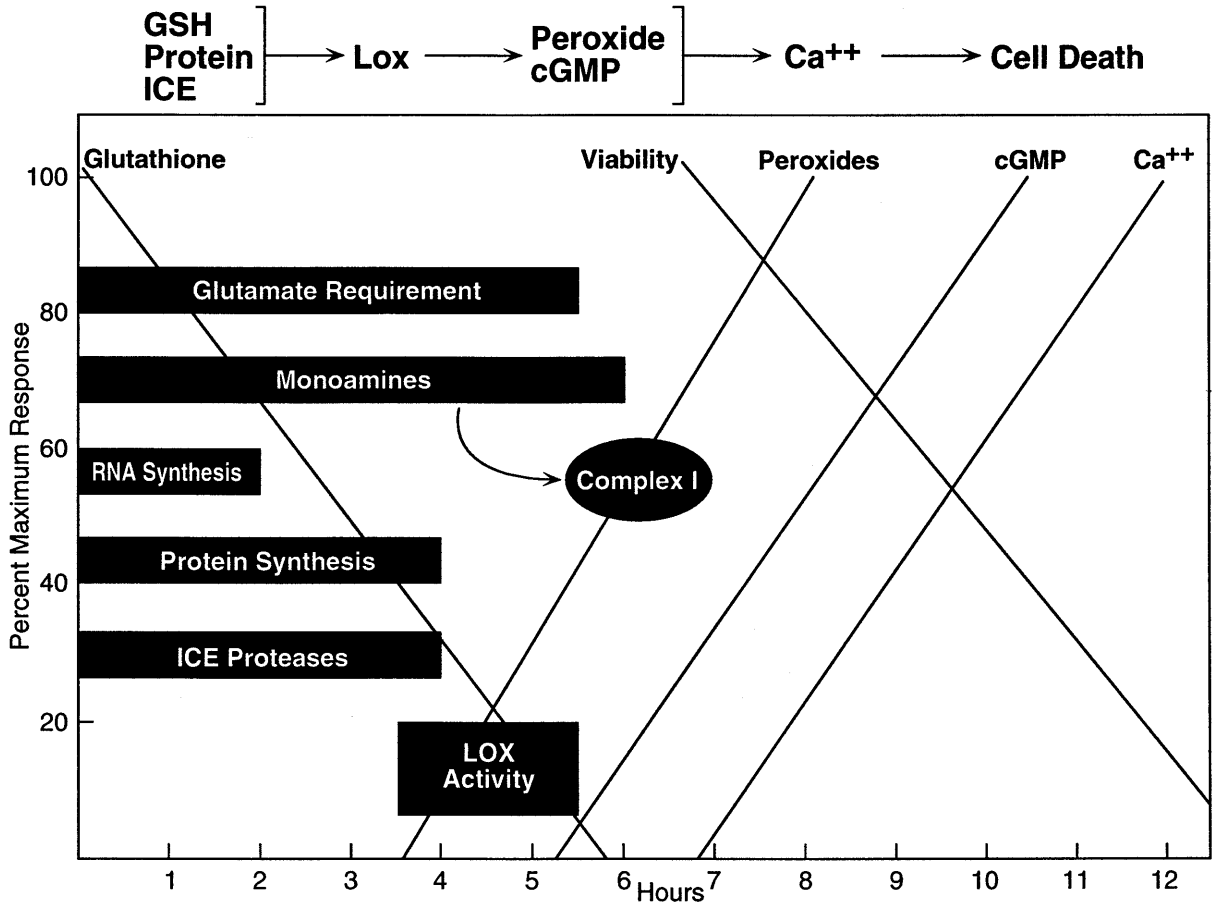

Figure 3. Schematic diagram of the time course of glutamate-induced cell death in rodent cortical neurons and the HT22 hippocampal nerve cell line. The solid lines indicate the relative increases or decreases in the molecules indicated and the solid rectangles indicate the times following the addition of exogenous glutamate when the indicated processes (e.g. RNA synthesis) occur. Monoamines are required for complex I in the mitochondria to generate ROS.

leaving cells viable for many hours in the presence of ROS at concentrations several hundredfold higher than normal—a condition most would view as catastrophic. The major downstream event from ROS accumulation which is required for cell lysis is Ca^{++} influx, which is to date the event closest in time to actual cell lysis which has been identified in oxidative glutamate toxicity. Ca^{++} influx occurs through a cGMP-activated Ca^{++} channel, and is required for cell death since death can be blocked by both cobalt, a nonspecific Ca^{++} channel inhibitor, and the removal of extracellular calcium. The function of this channel with respect to both Ca^{++} influx and cell death is also blocked by sGC inhibitors and inhibitors of ROS production [49]. Therefore, the accumulation of very large amounts of intracellular ROS is not sufficient to cause cell death, but it is a necessary step in the cell death process.

Finally, as stated above, in the oxidative glutamate toxicity cell death pathway there is no apparent decrease in mitochondrial membrane potential before complete failure, nor is there a release of cytochrome c. These data are at odds with most published accounts of apoptosis which show that there is a decrease in mitochondrial membrane potential. This membrane potential is established by the asymmetric distribution of protons and other ions across the inner mitochondrial membrane, and its loss frequently is associated with an irreversible commitment to cell death [50]. It has frequently been argued that the loss of the mitochondrial membrane potential is triggered by the sudden permeability increase of the inner mitochondrial membrane (permeability transition, PT), resulting in the leakage of proteins and the generation of ROS [51]. However, this does not occur in oxidative glutamate toxicity, again demonstrating the great diversity of mechanisms used by cells to carry out death programs.

ROS as signaling molecules

Although ROS have the potential to damage various intracellular macromolecules such as proteins, lipids and DNA, they also induce a variety of signaling pathways (for reviews see [52–56]). Whereas much of the research on these signaling pathways has been directed towards documenting a role for ROS in cell death (see earlier sections), there is also evidence that ROS can activate signaling pathways that lead to the induction of cell proliferation (e.g. [57–60]). The degree of overlap between the signaling pathways activated by pro-growth and pro-death conditions is not clear at this time. However, in general the activation of any type of signaling pathway by ROS involves the direct modification of a protein or other element of the signaling pathway by ROS. As described below, the consequences to cellular function may involve both the reversibility of the reaction and the location within the cell in which it occurs. In the following paragraphs a number of the proteins and signaling pathways affected by ROS will be described and the possible consequences of the modification of these pathways by ROS will be discussed. Studies which have utilized neurons or glial cells are specifically indicated. However, many of the studies utilized other types of cells, and effects of ROS on these proteins and signaling pathways in the cells of the nervous system remain to be determined.

Many of the effects of ROS may be mediated by reversible effects on intracellular proteins which lead to alterations in intracellular signaling pathways. Since the main intracellular antioxidants such as glutathione and Trx are thiol-containing molecules or proteins whose antioxidant activity is mediated by the reversible oxidation/reduction of cysteine sulfhydryl groups, it is likely that many of the direct effects of ROS on proteins are mediated by sulfhydryl groups in these proteins. For example, H_2O_2, which has been implicated in both oxidative stress and cell signaling, is a relatively mild oxidant that can oxidize protein sulfhydryl groups to produce cysteine sulfenic acid (CysS-OH) or disulfide bonds, both of which can be readily reduced back to cysteine by various intracellular reductants. However, under more severe and/or prolonged oxidative conditions sulfenic acid can undergo further oxidation to either sulfinic ($CysS\text{-}O_2H$) or sulfonic ($CysS\text{-}O_3H$) acid, both of which are irreversible. Despite the presence of cysteine in large numbers of proteins, only certain proteins are likely to be affected by this process since the oxidation of a protein sulfhydryl by H_2O_2 is dependent upon the pK_a of the target cysteine. For oxidation to occur, the pK_a of the target cysteine must be below 7.0, whereas the pK_a values of most cysteine resides in proteins is greater than 8.0 (see [54], and references therein). Although protein methionine residues can also be reversibly oxidized by H_2O_2, at the present time this amino acid has not been identified as playing a critical role in protein signaling. In addition, H_2O_2 and other oxidants may affect essential metal ions in specific proteins, changing their oxidation state and thereby altering their interaction with the protein. However, currently there is little direct evidence for this mode of action.

As discussed below, certain proteins such as protein tyrosine phosphatases and thioredoxin contain essential cysteine groups in their active sites which have low pK_a's, and their activities have been shown to be regulated by the cellular redox status. As more structural information becomes available regarding proteins involved in various signaling cascades, it should be possible to identify additional proteins whose activity is regulated by oxidation/reduction. These proteins are

likely to play important roles in the various responses of cells to changes in their redox status, and a characterization of their responses to oxidation could help define pathways involved in various aspects of cellular metabolism, including the response to oxidative stress.

Effects of ROS on protein kinase activity

ROS have been shown to increase the activity of both protein tyrosine kinases (PTKs) and serine/threonine kinases. However, in many cases it is not clear whether this is a direct effect on the kinase itself, an effect on an upstream activator or an effect on downstream protein phosphatases (see below). Since several recent reviews contain extensive lists of protein kinases which can be activated by ROS [55, 56, 61], we will focus instead on representative examples from each class of protein kinase and within a class, each potential mode of activation. Both receptor and nonreceptor PTKs can be activated by ROS. In both cases, the activation appears to be due mainly to an effect on downstream phosphatases (see below). For example, the treatment of cells with a variety of agents, including oxidants such as H_2O_2, was shown to induce tyrosine phosphorylation of the EGF receptor (EGFR) and PDGF receptor (PDGFR) by a mechanism involving the inhibition of dephosphorylation [62] rather than a direct effect on kinase activity. Indeed, treatment with H_2O_2 induced preferential phosphorylation of the EGFR on tyrosine, whereas ligand-activated receptor shows tyrosine, serine and threonine phosphorylation [63]. Furthermore, in many of these studies, whereas the phosphorylation of the receptors appeared to result in functional activation as determined by the binding of downstream substrates to the receptor, the actual effects of phosphorylation on receptor kinase activity were not determined.

A number of nonreceptor tyrosine kinases such as $pp60^{src}$ [64] and $p56^{lck}$ [65, 66] are shown to be activated by treatment of cells with H_2O_2 or other oxidants as determined both by increases in protein tyrosine phosphorylation and in vitro kinase activity. However, in the case of $p56^{lck}$, this activation could not be mimicked by direct treatment of the kinase with H_2O_2 [65], suggesting that the effects of ROS are indirect and probably mediated through the inhibition of phosphatase activity. In contrast, we have detected a direct effect of H_2O_2 on $pp60^{src}$ activity in immunoprecipitates, suggesting that in some cases ROS may be able to directly affect tyrosine kinase activity [unpublished]. Indeed, a number of years ago, Ltk, a nonreceptor, membrane-associated tyrosine kinase was described [67] which localizes to the endoplasmic reticulum and whose activity appeared to be regulated by the redox status of the cell. Dimerization through oxidation of specific sulfhydryl groups led to a significant activation of the kinase as determined by both in vitro kinase assays and anti-phosphotyrosine Western blots. Although this result could be due to an effect on phosphatases, the association of the majority of kinase activity with the dimerized protein, along with the lack of activation of the EGFR family member Neu under the same conditions, suggests that this tyrosine kinase may indeed be directly regulated by ROS. There is also some evidence that the PDGFR may be regulated by the redox status of the cell. Rigacci et al. [68] showed that the cellular GSH level specifically affected the tyrosine phosphorylation of the PDGFR in fibroblasts. These effects of ROS on PTK phosphorylation and/or activity may be cell type dependent. For example, H_2O_2 was found to specifically induce tyrosine phosphorylation and activation of $p72^{syk}$ in B lymphocytes, whereas in contrast to the studies described above, it had no effect on $p56^{lck}$ [69]. Treatment of cells with H_2O_2 also stimulates the tyrosine phosphorylation of the focal adhesion kinase, $pp125^{FAK}$. However, in contrast with the other PTKs discussed above, the phosphorylation of this kinase does not correlate with its activation. Furthermore, the phosphorylation of $pp125^{FAK}$ was not inhibited by several different PTK inhibitors [70]. For the most part, the consequences for the cell of PTK activation by oxidants is not clear. However, at least in some cases it appears to be part of a protective response. For example, ultraviolet (UV) irradiation of HeLa cells results in the ROS-dependent activation of the nonreceptor PTK $pp60^{src}$, whereas inhibition of $pp60^{src}$ activity potentiates cell killing by UV [64].

ROS also affect the activities of serine/threonine kinases. Perhaps the best known of these are members of the mitogen-activated protein kinase (MAP kinase) family. These serine-threonine kinases are activated by dual phosphorylation in response to a variety of extracellular stimuli. In mammalian cells, at least three distinct members of the MAP kinase family are expressed: ERKs (also known as MAPKs), stress-activated protein kinase (also known as c-Jun NH2-terminal kinase (JNK)) and p38 MAPK. ERKs are activated by growth factors and are primarily involved in cell proliferation and differentiation, whereas JNK and p38 MAPK are primarily activated in response to proinflammatory cytokines and environmental stress and are implicated in inflammatory responses, cell cycle arrest, DNA repair and cell death. The standard pathways for activation of these protein kinases consist of two upstream protein kinases: MAPKKKs, which phosphorylate and thereby activate MAPKKs, which phosphorylate the MAPKs on both a threonine and a tyrosine residue (for reviews see [71–74]). All three members of this family, including ERKs [75, 76], can be specifically activated by H_2O_2 and other oxidants, as determined both by dual phos-

phorylation and in vitro kinase assays. The specific ROS which lead to ERK activation may be cell type dependent since, in some cases, activation is seen with H_2O_2 [75], whereas in others it is seen with superoxide but not H_2O_2 [76]. ERK activation by H_2O_2 appears to be indirect since it is blocked by a dominant-negative Ras mutant, suggesting that H_2O_2 activates an upstream member of the prototypical Ras-Raf-MEK-MAPK pathway. Indeed, some reports suggest that Ras is responsive to the redox status of the cell and can be directly activated by ROS such as H_2O_2 [77] or nitric oxide [78]. A role for ERK activation in inhibiting cell death induced by a variety of stimuli has been demonstrated in a number of studies in a variety of different cell types including neuronal cells (e.g. [75, 79–82]).

Another, recently described member of the MAPK family, ERK5 or BMK1, is also activated by ROS [83] but through a very different pathway. In the case of ERK5, the activity of the PTK $pp60^{src}$ appears to be essential for ERK5 activation by H_2O_2 [83]. Other members of the src family of PTKs cannot substitute for $pp60^{src}$. Interestingly, in apparent contrast to the other members of the src family, $pp60^{src}$ can be directly activated by H_2O_2 (see above).

Several alternative mechanisms have been identified for the activation of the two other members of the MAPK family, JNK and p38 MAPK, by ROS. For example, JNK is generally thought to be activated through a pathway involving small GTP-binding proteins and a series of protein kinases terminating in the dual specificity kinases MKK4 and/or MKK7. However, recently Meriin et al. [84] have demonstrated that certain stimuli, including oxidative stress induced by a variety of agents, activate JNK not through this cascade but via inactivation of the phosphatases which generally maintain this kinase in a dephosphorylated and, therefore, inactive state. A second mechanism for the activation of both JNK and p38 MAPK by oxidative stress has also been described. ASK1 is a MAPKKK which can stimulate pathways leading to both p38 and JNK activation [85]. ASK1 activation is implicated in the promotion of programmed cell death [85, 86]. ASK1 activity in turn is regulated by the redox status of the cell. Two mechanisms for this regulation have been described. In one case [86], activation of ASK1 was brought about by H_2O_2-induced dimerization of the protein. Similar activation could be induced by synthetic dimerization. In the second study [85], activation of ASK1 was dependent upon Trx, a redox-sensitive regulatory protein (for review see [87]). Trx was found to associate with the N-terminus of ASK1 in a redox-sensitive fashion, resulting in inactivation of the kinase. Treatment with H_2O_2 or other oxidants resulted in the oxidation of Trx and its release from ASK1, leading to the activation of JNK and p38 MAPK. These two observations may not be at odds if the loss of Trx binding leads to the dimerization of ASK1. However, the role of ASK1 in either JNK or p38 MAPK activation may be cell type dependent since it is not the major MAPKKK for these kinases in many types of cells. A third mechanism for the regulation of JNK activity involves yet another protein involved in the maintenance of the cellular redox potential. Recently, glutathione *S*-transferase Pi (GSTp) was identified as a specific inhibitor of JNK [88]. An increase in ROS levels in cells led to GSTp oligomerization, dissociation of the GSTp-JNK complex and an increase in JNK activity. Furthermore, fibroblasts from GSTp-null mice had high levels of basal JNK activity relative to their normal counterparts which could be reduced by transfection with GSTp. In contrast with ERKs, the role of JNK in programmed cell death is controversial. Initial evidence suggested that high levels of JNK activity contributed to cell death, whereas inhibition of JNK activation was protective [79]. However, later studies have demonstrated a protective role for JNK activation in several different cell death paradigms (e.g. [89, 90]). One complicating factor is that the JNK family is encoded by three different genes (*Jnk1*, *Jnk2*, *Jnk3*) with alternative splicing giving rise to 10 different isoforms (4xJNK1, 4xJNK2, 2xJNK3) (for reviews see [72, 73]). A number of studies suggest that the different JNK isoforms may not be functionally redundant within cells. For example, recent studies with mice deficient in JNK1, JNK2, JNK3, or several combinations thereof, indicate distinct roles for the different isoforms in neuronal programmed cell death [91]. Whereas JNK3 knockout mice show an increased resistance to kainic acid-induced seizures and subsequent programmed cell death of hippocampal neurons, mice deficient in both JNK1 and JNK2 have a severe dysregulation of neuronal programmed cell death during embryonic development. Specifically, whereas these mice show a decrease in neuronal programmed cell death in the hindbrain, greatly increased neuronal programmed cell death is observed in the forebrain. The role of p38 MAPK in programmed cell death is slightly less controversial. Using specific p38 MAPK inhibitors, a number of studies have demonstrated that inhibition of p38 MAPK activity promotes cell survival in both neuronal [92, 93] and nonneuronal [94, 95] cells following exposure to a variety of stressful conditions.

The PKC family is a heterogeneous group of phospholipid-dependent serine/threonine kinases that are thought to be key elements in signaling pathways which regulate a wide range of cellular functions, including cell survival. The rapid loss of PKC activity is considered a prognostic feature of lethal damage to neurons following ischemia in vivo and hypoxic and excitotoxic insults in vitro (for reviews see [96–99]). The PKC family at present contains 11 different members which

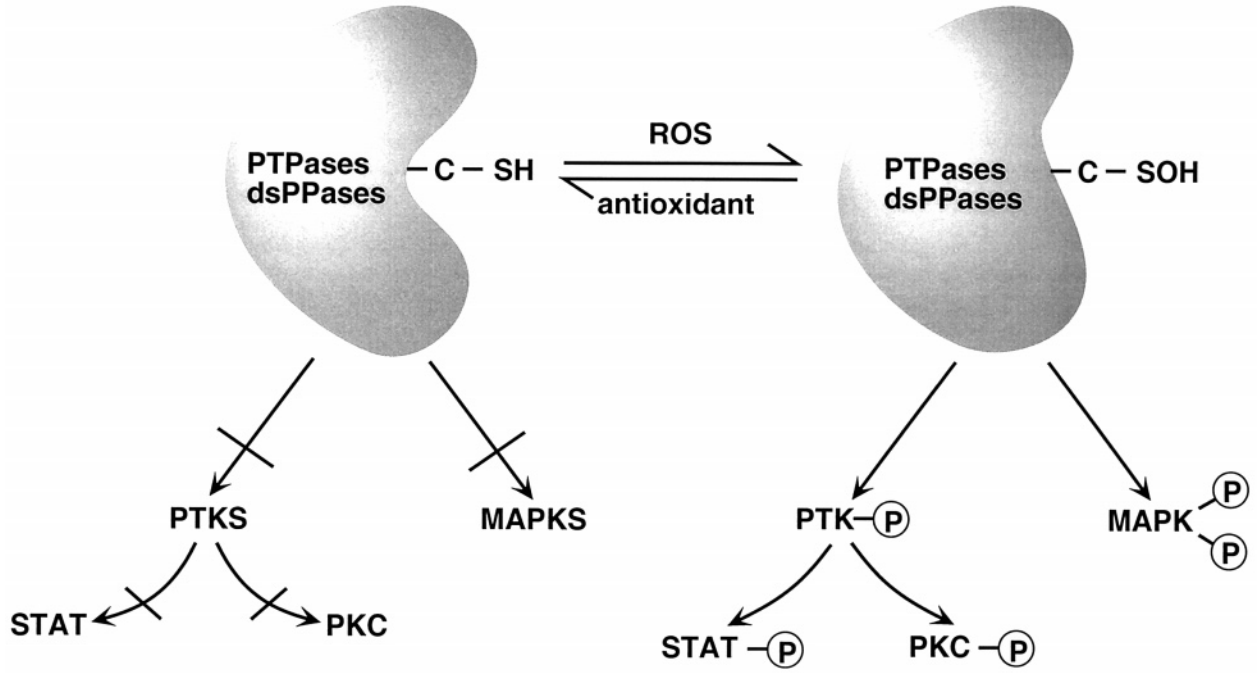

Figure 4. Regulation of protein phosphatase activity by H_2O_2. Both protein tyrosine phosphatases (PTPases) and dual specificity phosphatases (dsPPases) can be reversibly inactivated by H_2O_2 through the covalent modification of an essential cysteine in the active sites of the enzymes. Normally, PTPase activity maintains protein tyrosine kinases (PTKs) and PKCs, as well as members of the STAT family of transcription factors, in a state of low phosphorylation which, in the case of PTKs, correlates with low activity. Similarly, both dsPPase activity and PTPase activity maintain MAPKs in an unphosphorylated or partially phosphorylated state, which is inactive. Inhibition of phosphatase activity by H_2O_2 results in the activation of these proteins through autophosphorylation and/or phosphorylation by upstream kinases.

can be divided into three groups on the basis of structure and cofactor requirements. All members of this protein family are both phosphorylated on tyrosine and activated by treatment of cells with H_2O_2 and other ROS [100]. The effect of ROS on PKC appears to be specific to this protein kinase family because other, related protein kinases were not phosphorylated by the same treatments. Although the effect of ROS on PKC is clearly indirect, it is not clear whether it is mediated by activation of upstream PTKs or inhibition of downstream phosphatases, or both, nor is it clear what the consequences are to overall cellular function.

Effects of ROS on protein phosphatases

As alluded to in the section on protein kinases, a major mechanism for the activation of protein kinases by ROS may be through the inhibition of specific phosphatases (fig. 4). This is because kinases are activated by phosphorylation, and since phosphatases generally have about $10\times$ faster reaction kinetics than kinases, normally the dephosphorylated and therefore inactive state of the kinase predominates. There are three major classes of phosphatases in cells: tyrosine phosphatases (PTPases), serine/threonine phosphatases (PPases) and dual specificity (Thr/Tyr) phosphatases (dsPPases). The best-understood effects of ROS on phosphatase activity are on the PTPases. Within their active sites the PTPases contain a cysteine residue which is essential for enzymatic activity because it participates directly in the dephosphorylation reaction [101]. This cysteine is also very sensitive to the redox status of the cell. Although indirect evidence in a number of studies suggested that PTPases are a major intracellular target of H_2O_2 and other ROS (e.g. [62, 102]), recent work in the laboratory of Denu [103] directly demonstrated that a variety of different PTPases could be directly and rapidly inactivated by treatment with low concentrations of H_2O_2. This treatment resulted in the conversion of the essential catalytic cysteine to a stabilized sulfenic acid intermediate. Although this reaction is readily reversible in the presence of reducing agents, the reactivation process is much slower than the inactivation process, so that inactivation can occur in the presence of reducing conditions such as might be present in the cell.

dsPases act specifically on the terminal members of the MAPK family (e.g. ERKs, JNKs and p38 MAPKs) (for review see [104]). They also contain an essential cysteine in their active sites so they would be expected to undergo the same type of regulation in response to H_2O_2 or other oxidants as PTPases. However, since the activity of each of the substrates of these phosphatases is dependent upon phosphorylation of both tyrosine and threonine residues, these kinases can be inactivated by PTPases and PPases as well. Indeed, there is good evidence that in vivo PTPases play a role in regulating MAPK activity [104]. The evidence for a role for PPases in regulating MAPK activity is more controversial. However, in a recent paper it was demonstrated that p38 MAPK activation by H_2O_2 in astrocytes was concurrent with the inhibition of PPase activity [105]. In addition, direct inhibition of a subset of PPases (PP1 and PP2A) by okadaic acid also resulted in activation of p38 MAPK, whereas inhibition of another PPase, calcineurin (PP2B), did not.

Unlike the PTPases, PPases do not contain an essential cysteine residue at their active sites, so that the mechanisms underlying their inactivation by ROS are likely to be different. Indeed, even which ROS can and cannot inactivate these phosphatases is unclear. The Denu laboratory [103] found that the same conditions that resulted in complete inactivation of PTPases had no effect on the activities of three distinct PPases, PP2C, λ phosphatase and calcineurin. However, an earlier study had found that SOD could protect calcineurin from inactivation [106] and was thought to do so by blocking the oxidation of iron in the active site of calcineurin by $O_2^{\cdot -}$. Similar to the other PPases, calcineurin contains a pair of metal ions at the active site which are important for both catalytic activity and the structural integrity of the phosphatase [101]. $O_2^{\cdot -}$ may cause oxidation of the iron group in the active site, thereby rendering the phosphatase inactive. Very recently, it was shown that calcineurin can also be reversibly inactivated by H_2O_2 [107]. This inactivation does not involve the metal ions at the active site but rather appears to result from the cross-linking of two cysteines which lie outside the active site. How this cross-linking results in inactivation of calcineurin remains to be determined. In addition, both PP1 and PP2A are inactivated by reaction with GSSG [108]. The production of this oxidized form of glutathione is dependent upon the redox status of the cell [109]. Since GSSG interacts with cysteine groups in proteins [110, 111], these data suggest that a cysteine outside the active site but within the catalytic domain of these phosphatases is critical for enzyme activity and can be reversibly modified in a manner dependent upon the redox status of the cell. Thus, while PTPases and dsPPases can be directly affected by ROS such as H_2O_2, the PPases may be affected both directly via their metal ions and indirectly via redox-dependent amino acid modifications. These findings suggest that the spectrum of phosphatase inactivation by ROS within a given cell type will depend upon both the specific oxidizing agent and the effects that this agent has on the various detoxifying systems within the cell. The ultimate consequences of phosphatase inactivation by ROS on cellular function are not clear at this time but are likely to be cell type specific since they will depend upon the spectrum of kinase pathways which are activated or inhibited.

Activation of transcription factors by ROS

A number of different transcription factors are activated by ROS. Surprisingly, rather than a single mechanism for activation, a range of activation mechanisms have been identified. These include both direct effects on the transcription factors themselves and indirect effects mediated by either regulatory binding partners or upstream signaling pathways. Furthermore, at least in some cases, the activation is specific for a single type of ROS. The following paragraphs detail a number of these activation mechanisms and the transcription factors that they affect.

The signal transducer and activator of transcription (STAT) factors were originally described as growth factor- and interferon-inducible DNA binding complexes (for reviews see [112, 113]). These transcription factors play a role in the regulation of many genes, including the c-fos protooncogene, caspases and the cell cycle regulator $p21^{CIP1/WAF1}$. Unlike other transcription factors, the STAT factors are phosphorylated on tyrosine residues when cells are treated with an appropriate stimulus. Following phosphorylation the STATs undergo homo- and/or heterodimerization via SH2-phosphotyrosine interactions and become competent to bind DNA. The STATs also translocate from the cytoplasm to the nucleus following activation. Several PTKs have been identified, including Janus kinases (JAKs) and Tyk, which can phosphorylate STATs. Recently, several groups showed that along with cytokines and growth factors, STATs could also be activated by ROS [114, 115]. In one study using rat-1 fibroblasts, STAT3 was shown to be specifically activated, as determined by both DNA binding activity and transcriptional activity, by H_2O_2 but not by agents which generate either superoxide or NO [115]. This induction was blocked by antioxidants. The activation appeared to be via the upstream tyrosine kinases JAK2 and Tyk, whose activity was also induced by H_2O_2. However, the mechanism for this induction is still unclear since whereas direct inhibition of PTPases can strongly activate STATs, the pattern of activation is quite different from that seen

with H_2O_2 [115]. However, it maybe that H_2O_2 can only inhibit a subset of PTPases which act on STATs and/or their upstream kinases. In the second study which used lymphocytes [114], STAT3 was also found to be specifically activated by H_2O_2, and this activation was enhanced in the presence of PTPase inhibitors. Intriguingly, phenanthroline, an iron chelator, also blocked STAT activation by H_2O_2, suggesting that hydroxyl radicals could play a role in this activation. Thus, ROS appear to selectively activate a specific member of the STAT family by a mechanism which involves the activation of upstream activating kinases. The effects of STAT3 activation by ROS on cellular function remain to be elucidated.

The ARE or antioxidant responsive element is found in the promoters of a battery of genes which encode antioxidant and detoxification proteins and is responsible for the coordinated transcriptional activation of these genes. Among the genes which are activated through the ARE are enzymes which can correct imbalances in the cellular redox state resulting from decreases in GSH and/or increases in ROS. Included in the five classes of compounds which act on this promoter are both redox-cycling agents such as quinones and prooxidants such as H_2O_2 and lipid hydroperoxides. The transcription factors which act on this promoter and a partial picture of the mechanism underlying the activation of these factors have been recently elucidated (for reviews see [109, 116]). The transcription factor which binds and activates the ARE is a heterodimer of a small Maf protein and Nrf2. Maf is required for the sequence-specific DNA binding of the factor, whereas Nrf2 induces the transcriptional activation. The ability of Nrf2 to induce transcriptional activation is regulated by agents which activate the ARE, indicating that Nrf2 is directly involved in transducing the ROS signal. In unstimulated cells Nrf2 is found predominantly in the cytoplasm bound to a recently identified protein called Keap1 which, by interacting with the cytoskeleton, appears to keep Nrf2 from migrating to the nucleus [117]. Upon stimulation, the repression of Nrf2 by Keap1 is released, and Nrf2 translocates to the nucleus where it can interact with Maf and induce activation of the ARE. Exactly how ROS or other activators release the repression of Nrf2 by Keap1 is not clear, nor is it clear whether Nrf2 migrates to the nucleus along with Keap1 or whether activation releases Nrf2 from Keap1.

The regulation of the transcription factor AP1 by ROS is quite complex and highly dependent upon the context in which the regulation is examined. However, the studies with this transcription factor provide an excellent example of some of the confusion in the field as well as the way that the experimental paradigm affects the outcome. In addition, recent work on the regulation of AP1 by the intracellular redox potential points to a novel mechanism for transcription factor regulation whose importance in overall protein regulation is only beginning to be recognized. More details on AP1 regulation by ROS can be found in a number of recent reviews (e.g. [55, 61, 118, 119]).

Unlike the other transcription factors discussed here, AP-1 is not a single transcription factor but rather a group of related, dimeric complexes composed predominantly of Jun homodimers and Jun-Fos heterodimers (for reviews see [120–122]). Both the Jun and Fos families contain multiple members resulting in quite a complicated group of transcription factors. The differences in the different complexes are not well understood, although there is evidence that different complexes show distinct effects on transcriptional regulation. Dimerization of these proteins occurs via leucine zipper domains and results in the formation of a bipartite DNA binding site. Dimerization is necessary but not sufficient for DNA binding, which is mediated by amino acids in the adjacent basic region of the proteins. The dimers interact with the tetradecanoic phorbol acetate (TPA)-response elements in the promoter regions of a wide variety of genes implicated in cell proliferation, tumor promotion and the cellular response to stress. Similar to the other transcription factors discussed here, the DNA binding domains and the transcriptional activation domains of Jun and Fos reside in distinct regions of the protein. Curiously, the activation domains of these two proteins are controlled by different signaling pathways. The activation domain of Jun is regulated predominantly by the JNK kinases (see above), which phosphorylate Jun at serines 63 and 73 by a two-step mechanism wherein JNK first associates with Jun and subsequently phosphorylates it. As discussed in the previous section on ROS and protein kinases, JNK kinase activity is regulated by ROS. Although the Fos activation domains are also regulated by phosphorylation, the kinases involved remain to be identified. However, there is evidence indicating that the transcriptional activity of AP-1 complexes can also be influenced by signaling through the ERK pathway, which is also regulated by ROS (see above) [122]. Interestingly, genetic analysis indicates that both c-Jun and c-Fos null mice have increased levels of cell death following UV treatment as compared with their wild-type counterparts. This suggests that AP-1 activation results in the synthesis of proteins which are important in protecting cells from UV-induced cell death [122]. However, other studies have suggested that AP-1 activation can promote cell death (e.g. [120, 121]). Thus, similar to the role of JNK activation in programmed cell death, the role of AP-1 activation is open to debate. The AP-1 complex is also regulated by the expression of the Fos and Jun proteins. However, the relationship between protein expression and transcriptional activa-

tion is not straightforward. Whereas H_2O_2 strongly induces Fos and Jun expression, it only weakly induces AP-1 activity as defined by both DNA binding and transcriptional activation. In contrast, antioxidants strongly induce both protein expression and AP-1 activity [118, 123].

Thus, it is clear from the above description that ROS can affect AP-1 activity at a number of different sites ranging from induction of the synthesis of Fos or Jun to controlling protein phosphorylation to regulating DNA binding. In whole cells, treatment with H_2O_2 or agents which produce ROS can result in the activation of AP-1 (see [55, 118, 123]). However, numerous studies have shown that in vitro ROS inhibit DNA binding by AP-1. The redox regulation of DNA binding by AP-1 was first noted 10 years ago by Curran et al. [124]. They showed that a conserved cysteine in the DNA binding domain of Fos and Jun could be reversibly oxidized, resulting in a complex with little or no DNA binding activity. Mutation of the cysteine to serine resulted in an increase in DNA binding activity and a loss of redox regulation. Interestingly, the oncogenic homologue of c-Jun, v-Jun, already has this substitution, suggesting that part of its transforming potential may be due to a loss of redox regulation. Recent studies on the nature of the oxidized cysteine from Lamas et al. [125] has uncovered a novel mode of redox regulation for AP-1. They set out to examine whether the DNA binding activity of c-Jun could be regulated by the ratio of reduced to oxidized GSH. In normal cells, the ratio exceeds 100, whereas in various models of oxidative stress the ratio drops to values between 10 and 1. Half-maximal inhibition of AP-1 DNA binding activity was found at a GSH/GSSG ratio of 10 and was shown to be due to the reversible S-glutathiolation of the conserved cysteine in the DNA binding domain of c-Jun. Although a reduction in the GSH/GSSG ratio also caused c-Jun dimerization through a conserved cysteine in the leucine zipper region of the protein, this dimerization did not affect DNA binding. Several mechanisms for mixed disulfide formation with GSH have been proposed [110, 111], but further work will be required to define the mechanism operating here. Interestingly, mixed disulfide formation while temporarily inhibiting DNA binding activity could have the long-term effect of protecting the protein against oxidative damage by preventing the irreversible oxidation of cysteines to sulfinates and sulfonates. The oxidation may be reversed by Ref1. Ref1 is a DNA repair enzyme which can also regulate AP-1 activity [61, 119] and is probably the AP-1 activity protein originally described by Curran et al. [124]. The reducing activity of Ref1 is regulated by Trx (see above).

NF-κB was one of the first transcription factors shown to be regulated by ROS and is often considered to be a primary sensor of oxidative stress in cells (for reviews see [3, 119, 126]). Furthermore, in neuronal cells, NF-κB is implicated in playing a critical role in resistance to oxidative stress [127]. NF-κB is a member of a family of proteins which can homo- and heterodimerize to form a complex capable of binding to DNA. The most common form of this complex is the p50/p65 heterodimer. In unstimulated cells, NF-κB is found in the cytoplasm associated with the inhibitory protein IκB. Although a number of IκB proteins exist, the regulation of IκBα is the best understood. Following treatment of cells with many different stimuli, IκB is phosphorylated by an IκB kinase (IKK) and subsequently degraded in a proteosome-dependent manner. The free NF-κB is now able to translocate to the nucleus where it can bind DNA and initiate transcription of a diverse array of target genes including cytokines and growth factors, oxidative-stress-related enzymes, antiapoptotic proteins and cell-adhesion molecules. Many treatments which activate NF-κB are known to increase the production of ROS, including UV light and TNF-α, and antioxidants generally block NF-κB activation by these treatments. In addition, direct addition of H_2O_2 to some, but not all, cell lines activates NF-κB. Further evidence for the critical role of ROS and specifically, H_2O_2, in NF-κB activation comes from studies on cells stably overexpressing either catalase or SOD [128]. While catalase blocked the appearance of the active form of NF-κB in response to treatment of cells with either TNF-α or okadaic acid, SOD did not. The effect of catalase overexpression could be reversed by the catalase inhibitor, 3-aminotriazole.

It is not known how ROS activate NF-κB. One pathway involves the degradation of IκB as described above, but it is not clear whether ROS directly or indirectly increase the activity of an IKK or, instead, inhibit the activity of an IκB phosphatase. A second pathway for NF-κB activation has also been described and may be the predominant pathway induced by some forms of oxidative stress. Tyrosine phosphorylation of IκB on specific residues can result in the release of IκB from NF-κB without the resulting degradation of IκB [129]. This mechanism appears to be particularly prominent following reoxygenation after hypoxia [129, 130]. Since oxidative stress can block PTPase activity (see above), it seems likely that this mechanism for NF-κB activation is due to the direct inactivation of a PTPase that normally maintains IκB in a dephosphorylated state. Indeed, it was shown that in Jurkat cells, NF-κB activation required the activity of $p56^{Lck}$ [129] whose activity is known to be indirectly enhanced by ROS through the inhibition of PTPases (see above).

Similar to AP-1, NF-κB in the nucleus requires a reducing environment for DNA binding activity. This suggests that the agents which induce oxidative stress and

NF-κB activation must do so locally within the cytoplasm and that severe oxidative stress which leads to oxidizing conditions within the nucleus as well as the cytoplasm might result in an inability of NF-κB to activate transcription.

In addition to the transcription factors discussed above, the activities of a number of other transcription factors have been shown to be regulated by the redox status of the cell (for reviews see [55, 61, 118, 123, 131]). In almost all the cases which have been examined, this regulation involves direct effects on critical cysteines in the DNA binding domains or transcriptional activation domains of the proteins. A number of these transcription factors show the same conflict as seen with AP-1 wherein ROS activate the transcription factor in whole cells but in vitro studies with the isolated factor show it to be inhibited by oxidation. Clearly, further studies are required to resolve this apparent contradiction. Furthermore, these types of results indicate that whole cell data are critical when evaluating the effects of ROS on a given transcription factor. Among the other factors which show this dichotomy are NF-κB and p53. Other transcription factors are inhibited by oxidation both in in vitro assays and in whole cells. These include Sp-1, Egr-1 and GR. Another set have only been examined in one or the other assay systems, including USF and Ets. Thus, ROS may play critical roles in regulating the activity of a large number of transcription factors. The precise consequences of an increase in ROS depend not only upon the nature of the transcription factor itself but also upon the conditions within a given cell. This is most apparent for those transcription factors which appear to require an oxidizing cytoplasm but a reducing nucleus. Subtle changes in this balance could dramatically affect the activity of these transcription factors.

Summary

In this review emphasis has been placed upon the role of ROS as agents which initiate specific signaling pathways rather than agents which nonspecifically and irreversibly damage intracellular macromolecules. Therefore, even when ROS production leads to cell death, this maybe due to the activation of one or more signaling pathways leading to metabolic changes in the cell which eventually bring about cell death, and not due to generalized cellular damage. In a given cell type, the same ROS can lead to a different series of downstream events depending upon the concentration of the ROS and the duration of exposure. Similarly, the delivery of prooxidant stress by different means also may not produce the same set of physiological responses, and the same concentration of ROS can have distinct effects in different cell types. It follows that attempts to pigeonhole the cellular response to a given ROS into one or two narrowly defined categories will inevitably lead to a great deal of confusion, and that each cellular response to a given stress must be considered unique until proven otherwise.

Acknowledgements. Research in the authors' laboratories was supported by grants from the National Institutes of Health and the Department of Defense.

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