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Forum Review

Lipid Rafts and Oxidative Stress–Induced Cell Death

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ABSTRACT

Reactive oxygen species (ROS) are generated in response to a number of physiologic or pathologic conditions. In addition to ROS produced extrinsically, a cell may produce ROS as a result of normal metabolism and signaling processes. When sufficient quantities of ROS are present within the cell, this oxidative stress may have profound effects on the cell, including the induction of cell death. Various signaling pathways are initiated in response to oxidative stress, through which the cell's demise is assured. Many of these signaling pathways involve cholesterol-enriched domains of the cell membrane known as lipid rafts. These lipid rafts are platforms for initiation or transduction of the signal and may modulate protein activity through a direct change in local membrane structure or by allowing protein–protein interactions to occur with higher affinity/specificity or both. Among the examples discussed in this review are death-receptor signaling, induction of membrane-associated tyrosine kinase activation, and activation of transient receptor protein (TRP) channels. Special attention also is given to the RIP1/TRAF2 pathway, which involves the downstream activation of the stress-activated protein kinase JNK. The activation of the JNK pathway plays a key role in the induction of cellular death in response to ROS. *Antioxid. Redox Signal.* 9, 1471–1483.

INTRODUCTION

It has long been known that the membranes of eukaryotic cells are more complex in nature than a simple lipid bilayer, and that some proteins interact more efficiently with lipids than others. However, despite this knowledge, the influence of different lipids within the membrane on protein–protein interactions was largely ignored for a long time. We now know that hundreds of lipid species and thousands of different proteins are present in the plasma membranes of cells, and that these vary extensively with cell and tissue type. It is no surprise, then, that the different biophysical properties of different lipid and protein species give rise to heterogeneities within the surface of the cell membrane that affect the behavior of various binding and enzymatic processes. Among these heterogeneities in the plasma membrane are the somewhat controversial microdomains called lipid rafts.

The concept of the lipid raft was a significant alteration of the Singer-Nicholson "fluid mosaic model" of model of the early 1970s, in which a random and uniform two-dimensional lipid bilayer exists as a fluid matrix and has little effect on the function of a small concentration of proteins imbedded in its surface (21). The original lipid-raft hypothesis developed in the late 1980s and formalized by the late 1990s was one of relatively large (although too small to be resolved by standard light microscopy), stable lipid domains that were slightly more ordered than the surrounding lipids (called liquid ordered *versus* liquid disordered) because of the packing of cholesterol with sphingolipids (21, 93). Furthermore, these were postulated to be freely diffusible throughout the entire cell membrane and regulate cell dynamics by means of simple exclusion or inclusion of proteins based on their interactions with the lipids (93). Most of the early data (and one could argue much of the current data in the literature) are based solely on the resistance of rafts proteins to detergent solubilization (Triton X-100 at 4°C), or depletion of cholesterol with β-methyl-cyclodextrin (MCD) (94). Although these techniques are useful in determining whether a molecule is likely to be a raft molecule, it is clear that the presence of a protein in detergent-resistant membrane fractions (DRMs) is not always correlated with its localization

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in rafts (51), and that cholesterol depletion has multiple effects on membranes in addition to raft disruption. Unfortunately, the use of different methods to identify lipid-raft proteins led to discrepancies as to the actual nature and definition of lipid rafts and caused some even to question their existence (61).

More recent data using a variety of other techniques, including homo- and hetero fluorescence resonance energy transfer (FRET), electron paramagnetic resonance (EPR)-spin labeling, single particle tracking (SPT), and single fluorophore video tracking (SFVT) experiments, as well as electron microscopy (EM) and computational modeling, suggest that rafts are real but are substantially different from those initially proposed (33). Some evidence now suggests that rafts, in the absence of crosslinking agents, are actually quite small in unstimulated cells (on the order of $<$15 nm, and perhaps as small as 1–2 nm), and some may be composed of very few molecules. These constitutive raft structures appear to be dynamic entities in which both the lifetime of the raft and the residency time of molecules within the raft may be much shorter than was previously postulated (both on the order of $<$0.1 ms) (33, 45). In contrast with the previous model, the current model suggests that lipids (and also rafts) are slowed in their long-range diffusion because of the submembrane actin cytoskeleton "fence" with transmembrane protein "pickets" attached to this fence, confining raft signaling to a specific membrane compartment for a longer time, before diffusion is able to move the signal proteins to a new area of the cell membrane (45). In addition, it is now clear that protein–protein interactions or crosslinkings are important in the formation of more stable and larger raft structures (19). Nevertheless, the formation of rafts is essentially facilitated by the presence of cholesterol (33, 45). Thus, whereas protein–protein interactions (and also the lipid anchor or transmembrane domain of proteins inserted in the membrane) may be required to organize the lipids in the raft to some extent, the lipids provide additional stability and specificity for these interactions within the raft. Therefore, whether the lipid raft is primarily lipid or protein initiated or stabilized or both, the newly defined lipid raft still serves a function in signaling by organizing the proteins and lipids that come into contact with each other, influencing their enzymatic processes and determining what and for how long these interactions and/or processes might occur (3, 45).

OXIDATIVE STRESS

Oxidative stress refers to an increased level of intracellular reactive oxygen species (ROS) within the cell above the typical levels that the cell normally experiences. Such a condition may be generated by increased generation of ROS by various mechanisms or by an impaired function of the antioxidant defense system, which is responsible for dealing with basal ROS levels (92). Oxygen free radicals or, more generally, ROS, are the products of normal metabolic and signal-transduction events within a cell but may also play a role in pathologic processes. Cellular redox states play an important role in such processes as aging, malignant tumor progression, type II diabetes, atherosclerosis, chronic inflammatory processes, ischemia/reperfusion injury, as well as several neurodegenerative diseases. Cell death caused by abnormal redox situations appears to be the underlying cause of Alzheimer disease, Parkinson disease, and the familial form of amyotrophic lateral sclerosis.

Several contributors to cellular ROS levels are known (see Fig. 1). The mitochondria are the major source of ROS when the cell produces energy through the transfer of electrons down a series of protein complexes from molecular oxygen to water. Although these processes are contained and controlled by the structure of the mitochondria, some electrons leak from the system (6), especially from complexes I and III (9), at a rate that has been estimated at between 2 and 5% (7). Treatment of cells with inhibitors of some of the electron-transport chain complexes in the mitochondria often results in an increased generation of ROS. This is primarily because the electron-transport chain has no complex downstream to pass on the electrons, so they are passed on to the available molecular oxygen to pro-

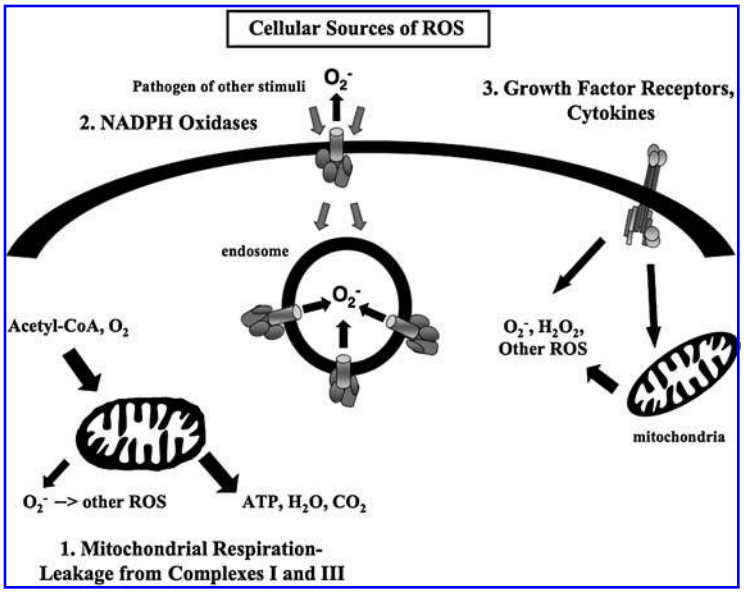

FIG. 1. Main cellular sources of ROS. The mitochondria are the major source of cellular ROS, especially through electron leakage from complexes I and III of the electron-transport chain. ROS are also produced by NAD(P)H oxidases in response to cellular pathogens and other activators. Growth-factor receptors and cytokines also produce ROS for use in their signaling pathways.

duce superoxide. Under most physiologic conditions, generated ROS are rapidly eliminated by antioxidant enzymes, including superoxide dismutases (SODs), catalase, glutathione peroxidases (GPxs), and peroxiredoxins (PRxs) (80).

A second major source of ROS in the cell is the NAD(P)H oxidase complex, which produces superoxide anions by the direct reduction of molecular oxygen by using NAD(P)H as a substrate (47). In this case, superoxide is used as both a signaling molecule and a weapon by the cell machinery. The so-called "oxidative burst" is characterized by massive production of ROS in an inflammatory environment and plays a key role in defense against environmental pathogens (77). In such an environment, activated neutrophils and macrophages produce large quantities of superoxide *via* the phagocytic isoform of NADPH oxidase. Originally thought to be a single enzyme, NADPH oxidases have now been found to comprise a large family of enzymes, with the expression of different isoforms being tissue specific (47). Various types of nonphagocytic cells, including fibroblasts, vascular smooth muscle cells, cardiac myocytes, and endothelial cells, are known to produce ROS by NAD(P)H oxidases to regulate intracellular signaling cascades (47, 77). On stimulation by growth factors and cytokines, NAD(P)H oxidases of vascular cells produce superoxide, which in turn can activate multiple intracellular signaling pathways (77). Some NADPH oxidases and their components are localized to lipid rafts, and it is thought that such require localization to these rafts for activation (107).

A variety of cytokines and growth factors that bind to receptors of different classes have been reported to generate ROS in nonphagocytic cells. Growth-factor receptors are receptor tyrosine kinases (RTKs) that play a key role in the transmission of information outside the cell into the cytoplasm and the nucleus. The information is transmitted in a variety of ways, including ROS and activation of mitogen-activated protein kinases (MAPKs) signaling pathways, or other second messengers. Activated growth factor–receptor signaling pathways that result in ROS production include epidermal growth factor (EGF) receptor, platelet-derived growth factor (PDGF) receptor, and vascular endothelial growth factor (VEGF) receptor (85). TNF-α, IL-1, and interferon (IFN-γ) were among the first reported cytokines to generate ROS in nonphagocytic cells (85). It is generally accepted that ROS generated by these ligand/receptor–initiated pathways can function as true second messengers and mediate important cellular functions such as proliferation and programmed cell death (85, 90). Thus, ROS play an important role in the regulation of cell function in many cell types. In addition, ROS play a regulatory role in cellular metabolic processes through the activation of various enzymatic cascades and regulation of several transcriptional factors.

CELL DEATH

Apoptosis and necrosis are often identified as two distinct forms of cell death. Apoptosis is often defined as a programmed cell death characterized by the activation of cysteine proteases known as caspases, which effect the downstream cellular damage. Apoptosis is associated with cellular shrinkage, chromatin condensation and nuclear fragmentation, membrane blebbing, and the formation of membrane-bound cellular bodies containing the contents of the now dead cell, which are then engulfed by phagocytic cells without much inflammation (24, 44). Necrosis is generally thought of as a passive death characterized by organelle and cellular swelling, dilation of cellular organelles (including large-scale mitochondrial damage and energy loss), and uncontrolled release of cellular contents due to loss of membrane integrity (24, 44). Although classically, necrosis has been described as accidental cell death occurring only in cases of severe pathologic damage, recent studies have revealed that necrotic cell death also occurs during normal cell physiology and development (20, 23). Autophagic cell death is a different form of programmed cell death that occurs after an attempt by the cell to survive by meeting its energetic needs through the lysosomal degradation of cellular proteins and organelles, and which requires specific genetically defined pathways (20, 44).

Apoptosis is often further subclassified based on how caspases are activated within the cell. Cell death triggered by direct caspase activation downstream of transmembrane "death receptors," such as Fas and TNF receptor-1 (TNFR1) in response to their ligands, is called the "extrinsic cell pathway." In this case, apoptosis is initiated through the recruitment of Fas-associated death domain (FADD) protein to the cytoplasmic death domains of an activated death-receptor complex, or in the case of TNFR1, a secondary complex that dissociates from the main complex (90). FADD induces dimerization and activation of the autocatalytic activation of the initiator cysteine proteases caspases-8 and -10 by direct recruitment of these caspases to its death-effector domain. Caspases then cleave intracellular substrates and result in cell death. In the "intrinsic pathway," cell death is triggered by regulated release of cytochrome *c* from the mitochondria, which binds to a complex containing Apaf-1 and caspase-9, resulting in the activation of this caspase. Release of cytochrome *c* is regulated by BH domain–containing proteins of the Bcl-2 family. Bax and Bak are the main proapoptotic members of this family that can trigger cytochrome *c* release and may be activated or inhibited by other BH proteins, which may be proapoptotic (activators, such as Bad, Bim, and Bid) or antiapoptotic (inhibitors, such as Bcl-2 or Bcl-xL). Cleavage of the Bid protein by caspase-8 allows it to activate Bax/Bak, providing a means of crosstalk between the extrinsic and intrinsic cell-death pathways.

Other "types" of cell death have been further defined based on the characteristics of dying cells (programmed or not) that are present or absent in specific cases, the causative agent or situation, and some types that represent "mixed cell death" with characteristics of both apoptosis and necrosis. Thus, we have in the literature such terms as oncosis (death through swelling, which is essentially the morphologic criterion of necrosis), paraptosis (activation of a nonapoptotic cell death that requires caspase-9, but not Apaf-1), anoikis (apoptosis that occurs after detachment), lipoptosis (death after a disturbance in fatty acid homeostasis), neosis (or mitotic catastrophe—a death that occurs after a failed mitosis), necroptosis (found as a Fas-dependent, but caspase-independent necrosis-like cell death), and aponecrosis (which is a death having apoptotic and necrotic features, likely after an incomplete execution of the apoptotic program followed by degeneration into necrosis). For simplicity's sake, we refer to only apoptosis and necrosis, with the realization that in many situations of cell death, especially when re-

lating to oxidative stress, cells have features of both, and possibly some kind of continuum exists between death types.

OXIDATIVE STRESS SENSORS AND EFFECTORS

The molecular targets of ROS in cell death have been largely elusive but are just beginning to be delineated. An important aspect of ROS biology is the multiple roles of ROS in cell death: ROS may act directly on cell death by straightforward oxidation of proteins, lipids, or nucleic acids, leading to a wide variety of cellular damage, or ROS can act as initiators or second messengers in the cell-death processes through various signaling cascades. In such a way, ROS can mediate the effects of many processes, such as treatment with cancer chemotherapeutic agents, UV, ionizing radiation, and tumor necrosis factor. Important issues regarding the role of ROS and oxidative stress in cell death remain to be studied further; however, it is known that ROS play a role in both apoptotic and necrotic cell death. In general, moderate oxidative radical stress induces apoptosis, whereas necrosis occurs when the cells are exposed to a higher dose of ROS. In most cases, in comparison to oxidative stress–mediated apoptosis, the signaling pathways controlling oxidative stress–induced necrotic cell death are poorly understood compared with the well-defined pathways of apoptosis.

Cell-death stimuli induce two different changes in mitochondrial function; the first is membrane potential disruption and permeability transition (PT), and the second is mitochondrial generation of ROS. Oxidative radical stress itself induces the generation of ROS in mitochondria, which acts as a positive-feedback loop. This model could explain several apparently contradictory data on the function of Bcl-2 in antioxidant pathways through a conditioning effect (43). However, typically Bcl-2 is thought of as inhibiting pro-oxidant–induced mitochondrial change and subsequent formation of ROS, and oxidative stress–induced response is thus suppressed by Bcl-2 in many cases (43). Diamide, a thiol oxidant that crosslinks thiols and thus mimics disulfide bond formation, induces mitochondrial membrane-potential disruption and permeability transition (14), whereas monovalent thiol-reactive compounds inhibit apoptosis induced by a variety of death stimuli (57). Thus, mitochondrial thiols constitute a critical sensor of the cellular redox potential. Thiols outside the mitochondria act as sensors of ROS as well, such as those found in thioredoxins (see later).

In addition to reaction with thiols, increasing evidence suggests that one of the major ways that ROS is sensed or by which it affects signaling pathways is through the direct reaction of these compounds with the catalytic sites of phosphatases. It has been known for some time that classic protein tyrosine phosphatases are inactivated by oxidation of their conserved catalytic cysteine by ROS (66). More recent data suggest that ROS are also capable of reacting with other phosphatases, such as dual-specificity phosphatases (40), which are capable of dephosphorylating both tyrosine and serine/threonine residues, and some of which can also remove phosphates from some phospholipids. Inactivation of the phosphatases by ROS may be both reversible or irreversible, depending on the oxidation state of the catalytic cysteine (16), and may lead to prolonged phosphorylation status (and activation) of many proteins within the cell, including those involved in kinase cascades, such as the stress-activated MAP kinases, p38 and JNK (16).

Several main pathways have been revealed to be involved in oxidative radical stress–induced apoptosis, and several more remain to be elucidated. The activation of JNK has a central role in many ROS-dependent apoptotic processes (54, 65, 89). In addition, ROS may induce apoptosis through several other defined pathways, including the production of ceramide, the activation of p53, and the induction of a regulatory protein of PI3-kinase, p85.

One group has identified an important relation between thioredoxin, a thiol-containing cellular redox regulatory protein, and ASK1 activation of JNK (82). These data show that the activity of ASK1 depends on the redox status of thioredoxin, and in its reduced form, thioredoxin is capable of binding to ASK1 and blocking its kinase activity (70, 82). Endogenous ASK1 forms a large complex, which has been termed the ASK1 signalosome (82). On stimulation with oxidant, thioredoxin is released, and TRAF2 or TRAF6 or both are recruited, forming an even larger complex that also contains downstream kinases and results in the activation of JNK (82). ASK1$-/-$ MEFs fail to induce sustained activation of JNK in response to H_2O_2, and these ASK1$-/-$ cells are resistant to H_2O_2-induced apoptosis (100). Therefore, it is believed that the ROS-thioredoxin-ASK1 system serves as an important molecular switch that converts redox signal to JNK kinase activation. In addition to ASK1 activation, JNK activation is positively affected by direct inactivation of JNK phosphatase activity by ROS (40). In addition, the monomeric form of glutathione S-transferase Pi (GSTπ) has been reported to bind directly to the C-terminus of JNK and inhibit its activation (111). ROS, such as H_2O_2, induce oligomerization of (GSTπ) and thus its dissociation from JNK, resulting in JNK activation (2).

Given that ROS induce activation of JNK cascade, apoptosis induced by ROS is sometimes dependent on a JNK-mediated mitochondrial-dependent apoptotic pathway. JNK is one of the two main groups of MAPK that are readily activated in response to various environmental stresses and plays a critical role in the processes deciding the fate of the cells. Substantial evidence supports the notion that JNK serves as an important proapoptotic mechanism in oxidatively stressed cells. First, JNK is readily activated by ROS *via* distinct signaling pathways, such as ASK1. Second, suppression of JNK by either genetic or pharmacologic approaches offers significant protection against apoptosis induced by ROS (89). Many studies have suggested that mitochondria are the main site of action for JNK in apoptosis. The most conclusive evidence is from a study using primary murine embryonic fibroblasts with deletion of both JNK1 and JNK2 (102). Further evidence supporting a role for mitochondria in ROS-mediated, JNK-dependent death is the observation that the Bcl-2 family, including Bcl-2 itself, is one of the main molecular targets of JNK and suggests that the proapoptotic activity of JNK is executed *via* regulation of Bcl-2 family members (65). Although other proapoptotic and also prosurvival functions of JNK are known, it is believed that the Bcl-2 family proteins are among the main molecular links between JNK and the mitochondria apoptotic machinery, as the proapoptotic Bax and Bak are required for JNK-dependent

apoptosis (50). Although much of the current literature addresses the proapoptotic function of JNK in apoptosis induced by ROS or oxidative stress *via* sustained JNK activation (54), accumulating evidence also suggests the importance of ROS-mediated JNK activation in nonapoptotic or necrotic cell death (89).

LIPID RAFTS, OXIDATIVE STRESS, AND CELL DEATH

Having discussed several of the basic mechanisms in ROS-mediated cell death signaling, we now move our discussion to the role of lipid rafts in ROS signaling. As most signaling pathways are interlinked at some point in the cell, and as therefore numerous raft molecules impinge on cell-death pathways in some way, we do not attempt a full analysis of all raftophillic molecules mentioned in the literature. Such would be impossible and not within the scope of this article. Instead, we attempt briefly to summarize what is known about several signaling molecules that are localized in, and are heavily dependent on, lipid rafts and/or caveolae, and that appear to be important players in ROS signaling and cell death, as examples of how some ROS and some lipid rafts may ultimately influence cell fate.

Death receptors

Extensive recent reviews discuss members of the large TNF superfamily and a subfamily of these receptors that contain a cytoplasmic death domain and are known as "death receptors." Some of these reviews address both the role of lipid rafts in activation/signal transduction and also the role of ROS in signaling in these receptors (23, 24, 42, 63, 73, 87, 90, 91). However, the roles of death receptors in ROS-mediated death cannot be completely passed over without some brief mention here, as they appear to play a role both upstream and downstream of ROS in many types of cell death (see Fig. 2).

Death receptors have been reported to be localized to lipid rafts in many instances, more especially Fas and TNFR1 (63), and cholesterol depletion changes the signaling events downstream of these receptors, suggesting that raft localization is important (49, 62). No doubt the localization of these receptors in rafts, or raft aggregates, is influenced by the way in which the receptors are activated by homotrimeric ligands, which likely stabilize the raft formation. Receptor aggregation would then

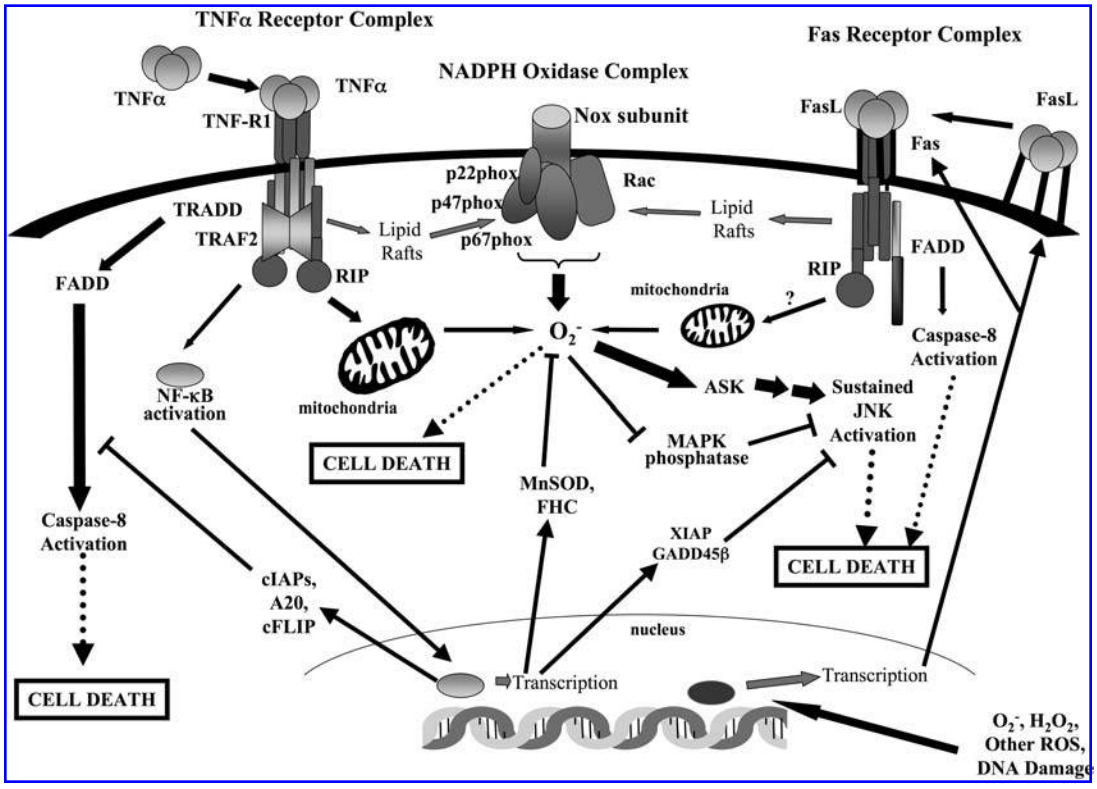

FIG. 2. Death-receptor pathways and ROS. The levels of death receptors and their ligands, such as Fas and Fas Ligand, may be transcriptionally induced by downstream effects of ROS or subsequent DNA damage. ROS also play a significant role downstream of death receptors in cell death, especially through the induction of sustained JNK activation, which is the result of ROS-induced kinase activation of upstream kinases such as ASK1. Sustained JNK activation is amplified by inactivation of MAPK phosphatases, which would normally reduce activated JNK levels. Death receptors may influence ROS levels by acting directly on the mitochondria or by activation of NADPH oxidases through initiating complex formation in lipid rafts. ROS may directly act on cell death by a direct reaction with protein, lipid, and nucleic acid cellular components, or may mediate effects as second messengers, leading to downstream death. In many cases, death is prevented by the activation of NF-κB, which blocks pro-death pathways as illustrated.

further stabilize the raft and allow raft aggregation and the recruitment of raftophilic molecules to the complex.

The role of these receptors downstream of ROS usually involves the transcriptional induction or stabilization of the receptors or their ligands. H_2O_2 or other oxidative stress has been shown to induce the expression of Fas and/or Fas ligand (FasL) mRNA or protein levels in Jurkat cells (5), NK cells (28), endothelial cells (95, 96), microglia (109), intestinal epithelial cells (17), and PC12 cells neuronally differentiated with nerve growth factor (NGF) (22). In a majority of these cases, apoptosis induced by ROS was dependent on Fas/FasL. Oxidized low-density lipoproteins (oxLDL) also induce apoptosis of activated T lymphocytes that is preceded by and dependent on an increase of Fas and FasL expression (3, 48). The DNA-damaging reagent cisplatin and γ-irradiation have been reported to induce ROS-dependent apoptosis by triggering Fas aggregation, whereas ROS scavengers effectively prevent the clustering of Fas receptor induced by these stimuli (36), suggesting that part of the role of ROS in initiating Fas-dependent apoptosis may be due to ROS facilitation of the formation of lipid rafts aggregates.

In search of the molecular mechanisms controlling death receptor-induced apoptotic and necrotic cell death, many studies have also demonstrated a role for ROS and oxidative stress downstream of death receptors (87, 90), especially for Fas and TNFR1. Both Fas and TNFR1 are known to generate ROS in response to stimulation. The production of ROS in many cases has been suggested to come from downstream events involving mitochondria (24). However, TNF is a well-known potentiator of NADPH oxidase in phagocytic cell types, and it is now clear that both TNFR1 and Fas can stimulate production of superoxide by using NADPH oxidase in nonphagocytic cell types (78, 118). Production of superoxide by this method may be dependent on the formation of lipid rafts (107), and colocalization of the receptor with NADPH oxidase components (118), which have been shown to localize to rafts (107), or relocalize to putative rafts on TNF treatment (114). Generation of ROS downstream of Fas and TNFR1 contributes to their ability to induce apoptosis or necrosis in many cases (18, 38, 87, 90, 97).

Although ROS has been linked to JNK activation for some time, it has been difficult to decipher the molecular mechanisms involved in this pathway on exposure to TNFα. With the recent understanding of the functional interplay between NF-κB and JNK (79, 65, 99), the proapoptotic function of ROS has now been appreciated, based on their mediating role between these two key signaling pathways downstream of the TNF receptor. Although the activation of the transcription factor NF-κB is important in preventing cell death by TNFα through the induction of inhibitory molecules (42), such as the cytoplasmic zinc-finger protein A20, the cellular inhibitor of apoptosis proteins (cIAPs), the antiapoptotic Bcl2 protein A1, and the caspase-8 inhibitory protein cFLIP, other NF-κB upregulated proteins may effect apoptosis by affecting JNK activation, such as XIAP and GADD45β (72, 99). Two phases of JNK activation in response to TNF that differ in how they affect cell death and also in their requirement for ROS generation. The first phase appears to be protective against cell death (46, 106) whereas the second sustained phase is mediated by ROS through ASK1 (see later), and positively affects both apoptosis and necrosis (12, 100, 106). TNF-α–induced NF-κB activation can prevent ROS production and accumulation *via* enhanced expression of antioxidants such as MnSOD and FHC (39, 75), and thus prevents sustained JNK activation (73, 79, 93). Therefore, ROS and oxidative stress occupy a unique position in cell death induced by TNF-α, and ROS scavenging molecules prevent cell death induced by TNF-α in many instances (24, 87, 90). Such observations provides credence to earlier reports linking TNFR-associated factors (TRAF) to ROS production TNF-α–treated cells (11) and the fact that TRAF2 is a critical adaptor protein in TNF-α–induced JNK activation (115). We recently reported that TNF-α–induced nonapoptotic cell death was evident only in wild-type MEF cells with high levels of ROS, but not in RIP$-/-$ or TRAF2$-/-$ MEFs, which lacked ROS accumulation after TNF-α treatment (52). Consistent with other findings that exogenously applied ROS induce necrotic cell death *via* JNK activation (see later) (88), JNK is now proposed as a critical mediator in TNF-α–induced necrotic cell death (90). ROS act as important coactivators in TNFR1-mediated JNK activation, and the sustained JNK activation constitutes one of the key events in both apoptotic and necrotic TNF-α–induced cell death (12, 40, 105, 106).

Activation of membrane-associated protein tyrosine kinases in oxidative stress

As mentioned earlier, the activation of some receptor tyrosine kinases (RTKs) has been shown to result in the generation of ROS that function as second messengers. However, RTKs and other tyrosine kinases, such as members of the Src family kinase, which can be linked to the membrane by myristoylation or palmitoylation of their N-terminal amino acids, are also activated by oxidative stress (see Fig. 3). First, ROS enhance the activation of kinases by inactivating the catalytic cysteines of the regulatory phosphatases that are usually recruited to the complexes concurrent with activation (16, 66, 67). Second, ROS appear to mediate a direct activation of these kinases. In the case of the RTKs, this activation is mediated independent of their ligands. Activation is proposed to be mediated by direct oxidation of specific cysteine residues that result in structural changes that favor an active conformation (29) or by the formation of specific intermolecular disulfide bonds with other molecules within the complex (66, 80). It is thought that lipid rafts play a role in the activation process, by causing the aggregation of the kinases and allowing their crosslinking (66, 80) and thus promoting activity, and also by the coupling together of ROS-generating oxidation machinery (localized within lipid rafts) with the ROS-sensitive cysteines required for activation (10). The downstream events after RTK or Src-family kinase activation are numerous; however, several pathways are worthy of mention. Tyrosine phosphorylation of caveolin-1 on Tyr 14 by c-Src kinases [possibly by c-Abl (84), downstream of Fyn (83)] regulates the trafficking/localization of caveolae in such places as focal adhesions (110), is required for caveolar internalization, and may thus regulate the composition/localization of certain lipid rafts (15). c-Src is proposed to be required for activation of JNK by H_2O_2 in several studies. Pharmacologic inhibition of Src or the use of a dominant negative Src mutant disrupted JNK activation by H_2O_2 in endothelial cells, vascular smooth muscle cells, and fibroblasts (13, 116), and cells derived from Src-deficient mice failed to activate JNK in response

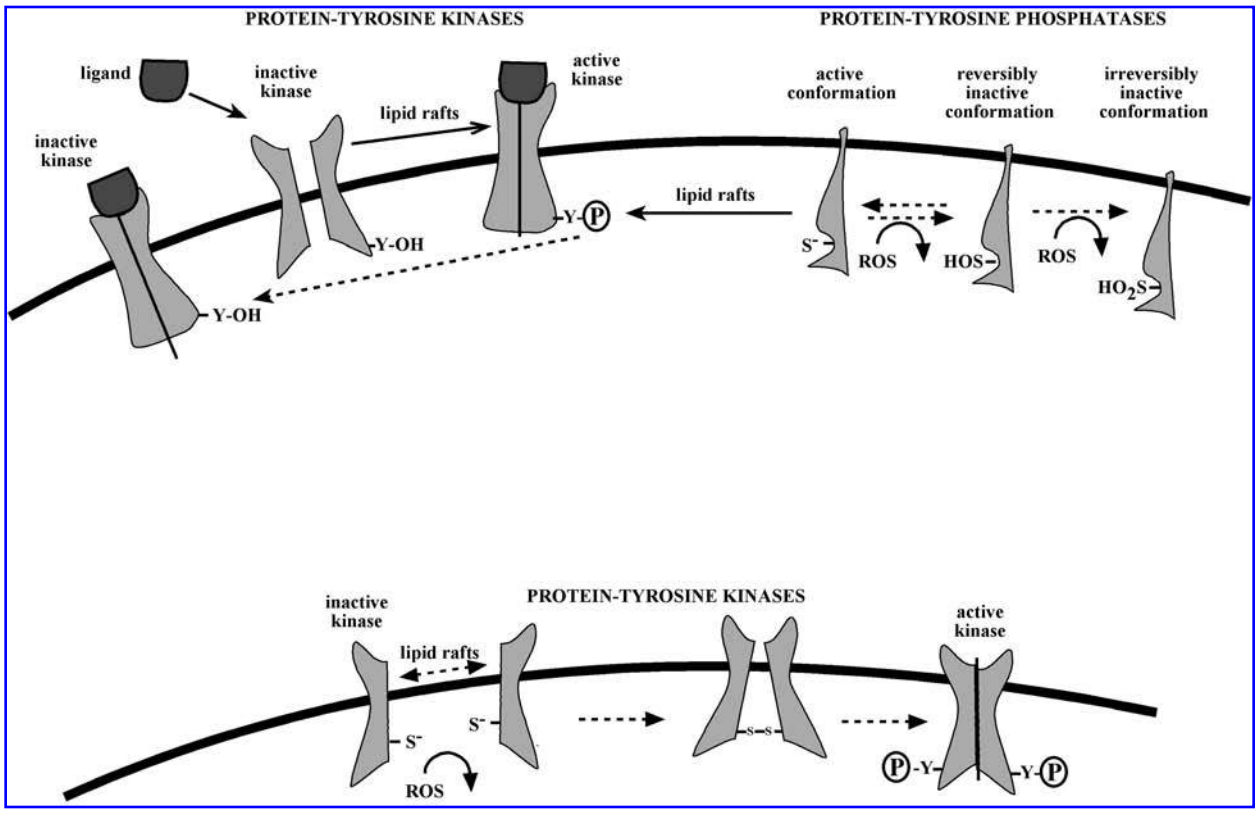

FIG. 3. Role of ROS in protein-tyrosine Kinase (PTK) activation. *Top:* ROS may temporarily inactivate a PTK phosphatase by reacting with the catalytic cysteine to form sulphenic acid. Further oxidation of the phosphatase by ROS leads to sulphinic (shown) and sulphonic (not shown) acid formation that irreversibly inactivates the phosphatase. In many cases, ROS has been shown to be necessary for the full activation of the kinase. *Bottom:* Alternatively, a PTK may be activated through a ligand-independent mechanism by inducing formation or rearrangement of intermolecular (shown) and intramolecular (not shown) disulfide bonds, resulting in conformational changes that result in kinase activity.

to H_2O_2, but p38 and ERK pathways were intact (116). Src family kinases also may contribute to membrane targeting of Fas in response to oxidative stress, a process that requires the phosphorylation of the EGF receptor (EGFR) and subsequent EGFR phosphorylation of Fas (78).

The role of TRP channels in oxidative stress and cell death

Transient receptor proteins (TRPs) are a family of calcium-permeable and voltage-independent cation channels that are divided into seven different subfamilies that comprise almost 30 different proteins. Many of these proteins have been shown to localize within lipid rafts or caveolae or both (4, 8, 31, 55, 56, 101) and act as sensors for a wide range of stimuli, including temperature (heat and cold), osmotic pressure, mechanical force, and other chemical and physical stimuli (108). Four of these channels are also known to be triggered by oxidative stress, and other channels in this family are likely to be sensitive to the cellular redox environment, but have not yet been examined in the context of this stimulus. As the literature is quite extensive on this subject, we briefly summarize of what is known about these receptors in their relation to oxidative stress, and for further information refer the reader to several more-detailed reviews that have been written within the past few years (58, 59, 68, 86, 108), some of which have specifically addressed the role(s) of TRP channels in the context of cell death (58, 59).

The four TRP channels that are known to be influenced by oxidative stress are TRPC3, TRPC4, TRPM2, and TRPM7. Whereas TRPC3 and TRPC4 are known to localize/relocalize to lipid rafts (4, 8, 31, 55, 101), it has not yet been definitively determined whether TRPM2 and TRPM7 do likewise. TRPC3 and TRPC4, being from the TRPC subfamily, are Ca^{2+}-permeable channels regulated by the products of phospolipase C, inositol 1,4,5-trisphosphate (IP3) (or both) and diacylglycerol (DAG) (TRPC3 only), in addition to redox. The channels may participate in Na^+ loading and may regulate calcium levels by a coupled Na^+/Ca^{2+} exhange process (81). These two channels can also form a TRPC3–TRPC4 complex with different properties from their respective homomeric channels, and that is also redox sensitive (76). Activation of TRPC3 by DAG is inhibited by dominant negative Src and pharmacologic inhibition of Src kinases (104), and it thus appears that tyrosine kinase activation plays a role in redox activation of TRPC3. However, in other experiments, the PLC inhibitor U73122 clearly inhibits oxidative stress–induced activation of TRPC3, demonstrating an essential role for phospholipase C (31). Although TRPC3

and TRPC4 are activated in response to oxidative stress, their exact role in cell death in consequence to Ca^{2+} influx has not yet been determined but is likely to have an impact. One of the most important consequences of increasing cytosolic Ca^{2+} concentrations with regard to cell death is that high Ca^{2+} concentrations promote the opening of the mitochondrial permeability transition pore (PTP), which results in the uncoupling of oxidative phosphorylation, bioenergetic catastrophe, and ultimately necrotic cell death (32). Many effects of Ca^{2+} concentrations on apoptosis have also been observed.

TRPM2 and TRPM7 have both been shown to have important roles in cell death due to oxidative stress in some cell types (58, 59, 86). Both of these channels have additional enzymatic intracellular domains in addition to their channel domains and are therefore sometimes referred to as "chanzymes" (86). However, the enzymatic activity of these domains from both of these proteins is not required for channel opening (86). TRPM2, which is permeable to Na, K, and Ca^{2+}, has an ADP-ribose hydrolase domain that binds to ADP-ribose (ADPR) and thus acts as a sensor for ADPR in opening the channel. TRPM7, which is permeable to Na^+, Ca^{2+}, and Mg^{2+}, as well as perhaps other divalent cations, has a Mg^{2+}-dependent Ser/Thr kinase domain, which may contribute to regulate channel opening in response to Mg^{2+} and play a role in regulating Mg^{2+} homeostasis (60). Both of these channels are opened in response to H_2O_2 and other oxidants, which results in a Ca^{2+} influx (58, 59, 86).

Anti-sense oligo experiments targeting TRPM2 in HEK cells transfected with TRPM2 or other cells (RIN-5F, U937) that expressed the endogenous protein established that TRPM2 was required for Ca^{2+} influx and death induced by H_2O_2, or by TNFα, which also causes ROS production (34). Expression of a dominant negative version of TRPM2 also blocked H_2O_2-mediated Ca^{2+} influx and cell death in HEK cells expressing TRPM2 (119), and it also protected rat striatal cells, which endogenously express TRPM2, from similar effects without affecting ROS production (26). In an elegant approach, Zhang *et al*. (120) modulated the levels of TRPM2 isoforms within U937-ecoR cells and showed that cells expressing the long form of TRPM2 were more sensitive to H_2O_2-mediated apoptosis, and that such was blocked in the presence of the Ca^{2+} chelator BAPTA, showing a critical role for Ca^{2+} in H_2O_2-mediated cell death through TRPM2 (120). Depletion of TRPM2 by using siRNA in cultured rat neurons suppressed the H_2O_2-induced Ca^{2+} influx, and significantly inhibited H_2O_2-induced death (41). All of these reports suggest that TRPM2 has an important role in oxidative stress–induced cell death. TRPM7 has not been as well studied but appears to function similarly in rat cortical neurons in response to H_2O_2 and other oxidative stress (1), although it is clearly involved in Mg^{2+} homeostasis (60) and is required for cell viability of some cell types (64).

It is not clear whether oxidants gate TRPM2 directly. The difficulty in determining this lies in the fact TRPM2 is gated in response to ADPR, which may be produced by the mitochondria in response to oxidative stress or by activation of a PARP-like enzyme that that hydrolyzes NAD to ADPR in response to oxidative DNA damage (59). In HEK cells, expression of a C-terminal mutant version of TRPM2 with a deletion in the region responsible for ADPR binding gave a channel that was sensitive to H_2O_2, but not to ADPR, suggesting direct activation by ROS (112). Conversely, inhibitors of PARP have been shown to block H_2O_2-mediated intracellular Ca^{2+} increase by TRPM2 and subsequent mitochondrial dysfunction, suggesting that ROS generates ADPR as a second messenger (25, 74).

As we are discussing cell death in the context of lipid rafts, it is likely that many different TRP channels may be directly gated or influenced by changes in the composition and packing of lipids around them, such as might occur when a nonraft protein relocalizes to within a lipid raft, or *vice versa*. Studies support the idea that mechanosensitive channels, such as some TPR channels (108), are capable of being conformationally influenced (and thus activated) by lipid composition within the surrounding lipid bilayer (113). [See Fig. 4 for a hypothetical example. The reader is also referred to Janmey and Kinnunen's excellent review on membrane dynamics for an explanation of how this may happen (37).] Indeed, the TRPC3 channel membrane conductance may be activated by cholesterol loading of the cell membrane when TRPC3 is overexpressed (30). As previously mentioned, many molecules relocate to or from lipid rafts on treatment with oxidative stress, and such a hypothesis

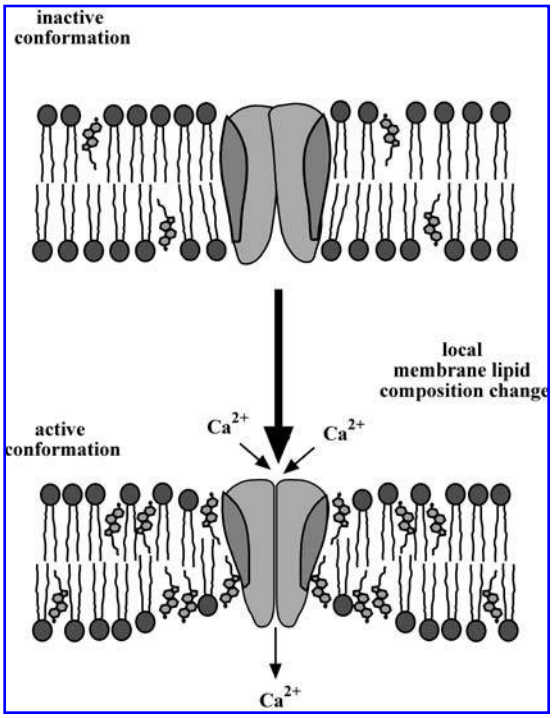

FIG. 4. Hypothetical model of the influence of lipid rafts on the gating of TPR channels. Membrane thickness varies around a transmembrane protein as it is influenced by the thickness of the exposed hydrophobic domain. The membrane likewise has effects on the protein. Changes in lipid packing due to local lipid composition (such as a lipid raft) around the protein result in a change in hydrophobic height and a decrease or increase in lateral pressure in the membrane around the protein. These changes may potentially change the protein conformation, resulting in an active protein. Thus, the change of localization of a membrane protein to within a lipid raft may result in its activation. Likewise, the removal of a protein from a lipid raft may induce similar changes, depending on the structure and hydrophobic regions of the protein.

could potentially explain the activation of the C-terminal mutant of TRPM2 that disrupts the affinity for ADPR, but still maintains H_2O_2 sensitivity.

The TRAF2/RIP-dependent pathway for oxidative cell death (88)

Our laboratory has shown that treatment of fibroblasts with moderate amounts of H_2O_2 induces a complex between TRAF2 and RIP that appears to be required for oxidative stress–induced cell death, which in this case is necrotic and zVAD-independent. TRAF2 and RIP are relocalized to lipid rafts (colocalization with GM1) on treatment with H_2O_2, resulting in downstream activation of JNK. Large raft conglomerates are apparent by following RIP by using immunofluorescence after 30 min of treatment with H_2O_2. This process is independent of *de novo* protein synthesis and takes place in the absence of the TNFR1 (DR3 and Fas were also excluded from involvement), because TNFR1−/− MEFs are as sensitive to H_2O_2 as are wild-type MEFs in our hands. [This is in contrast to another report that suggested TNFR1 involvement in H_2O_2-mediated JNK activation in lung fibroblasts from TNFR1−/− mice (71).] However, RIP−/− or TRAF2−/− MEFs fail to undergo cell death in response to H_2O_2. Replacement of RIP or TRAF2 in their respective knockout cells restores sensitivity to oxidative stress imposed by H_2O_2. Similar results also are obtained in RIP-deficient Jurkat cells, which apparently lack caveolin-1 and caveolae (27, 35), indicating that the lipid rafts formed in response to oxidative stress that contain RIP and TRAF2 are noncaveolar in nature. Interestingly, we found that FADD−/− MEFs are highly sensitive to oxidative stress, and reduction in H_2O_2 sensitivity returns to normal levels when FADD expression is restored to the FADD−/− cells. This may imply a certain level of basal cleavage of RIP by a FADD-dependent caspase-8–mediated process, as has been suggested in some cell types (117). This is supported by the fact that zVAD treatment increases the sensitivity to H_2O_2 treatment in our hands. Alternatively, FADD has been shown to bind directly to RIP under some circumstances and may directly inhibit RIP-TRAF2 complex formation (103).

In our hands, JNK activation plays an essential role in the TRAF2/RIP-dependent pathway for oxidative cell death in response to H_2O_2. The JNK1 isoform, but not the JNK2 isoform, appears to be required, because JNK1−/− MEFs fail to die in response to H_2O_2, but not JNK2−/− MEFs. In addition, a pharmacologic JNK inhibitor blocks JNK activation and prevents H_2O_2-mediated necrotic death in wild-type MEFs. However, a conflicting report suggests that JNK1−/− JNK2−/− cells are more sensitive than wild-type MEFs to H_2O_2 treatment (105). Nevertheless, we found that JNK1 (but not JNK2) actually coimmunoprecipitated with TRAF2/RIP in H_2O_2-treated cells and colocalized in lipid rafts, suggesting a somewhat direct activation mechanism. Curiously, the kinase activity of RIP was not required for JNK activation, and the upstream kinases MEKK1, MKK4, or MKK7 were not detected in the coprecipitate. Given the proposed role of ASK1 in oxidative stress–induced death, its activation by ROS, its well-known binding of TRAF2 during TNF, ER-stress, and oxidative stress–mediated pathways, ASK1 would seem a logical choice for activation of JNK in lipid rafts (53, 69, 70, 82, 100). However, this would probably involve an additional unknown kinase between ASK1 and JNK1, as direct phosphorylation of JNK by ASK1 in the absence of MKK4/MKK7 is unlikely. Could the large ASK1 signalosome that was induced after H_2O_2 treatment by Noguchi *et al*. (70) be in the same complex that we found in lipid rafts? Given that this complex is proposed to contain both TRAF2 and TRAF6 (70), this is a distinct possibility, but has yet to be tested. Clearly, further investigation into the role of lipid rafts in RIP/TRAF2-meditated oxidative stress is desirable. Besides investigating a possible role for ASK1 or the ASK signalosome or both in this pathway, it will be especially important to determine how raft targeting/localization of RIP and TRAF2 is achieved and what other signaling molecules the raft may contain.

CONCLUSIONS

Oxidative stress may trigger different kinds of cell death *via* many different signaling pathways. As has been shown in this review, many of these signaling pathways involve the cholesterol-enriched lipid rafts of the cell membrane, which may mechanically or biochemically alter the enzymatic activity or channel gating of important signaling proteins, resulting in the initiation or the continuous transduction of a cell-death signaling pathway. Lipid rafts play an important role in death-receptor signaling, induction of membrane-associated tyrosine kinase activation, activation of TRP channels, and transduction of the RIP1/TRAF2 pathway, which involves the downstream activation of JNK. However, the understanding of lipid-raft signaling with regard to these pathways is still in an early stage, and much remains to learn about the individual contribution and cooperation between these pathways as they contribute to induction of cellular death in response to oxidative stress.

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ABBREVIATIONS

ASK1, Apoptosis signal–regulating kinase 1; ADPR, ADP-ribose; cFLIP, cellular FLICE-like inhibitory protein; cIAPs, cellular inhibitor of apoptosis proteins; DR3, death receptor-3; DRM, detergent-resistant membrane fractions; EGF, epidermal growth factor; EGFR, EGF receptor; EM, electron microscopy; EPR, electron paramagnetic resonance; ERK, extracellular signal-regulated kinase (MAP kinase-1); FADD, Fas-associated death domain; FasL, Fas ligand; FHC, ferritin heavy chain; FRET, fluorescence resonance energy transfer; GADD45β growth arrest and DNA damage 45 beta; GM1, monosialotetrahexosylganglioside; GPxs, glutathione peroxidases; GSTπ glutathione *S*-transferase Pi; IFN-γ: interferon gamma; IL-1, interleukin-1; JNK, Jun-N-terminal kinase; MAPKs, mitogen-ac-

tivated protein kinases; MCD,β-methyl-cyclodextrin; MEFs, murine embryonic fibroblasts; MEKK1, mitogen-activated protein kinase kinase kinase-1; MKK4, map kinase kinase-4; MKK7, map kinase kinase-7; MnSOD, manganese superoxide dismutase; NF-κB, nuclear factor kappa-B; NGF, nerve growth factor; oxLDL, oxidized low-density lipoprotein; PARP, poly(ADP-ribose) polymerase; PDGF, platelet-derived growth factor; PLC, phospholipase C; PRxs, peroxiredoxins; PT, permeability transition; PTP, mitochondrial permeability transition pore; RIP1, receptor-interacting serine/threonine-protein kinase-1; ROS, reactive oxygen species; RTKs, receptor tyrosine kinases; TNF-α tumor necrosis factor alpha; TNFR1, TNF receptor-1; TRAF2, TNF receptor–associated factor 2; TRAF6, TNF receptor–associated factor 6; TRP, transient receptor protein (channels); TRPC, transient receptor potential-classic (channels); TRPM, transient receptor potential-melastatin (channels); SFVT, single fluorophore video tracking; SODs, superoxide dismutases; SPT, single-particle tracking; XIAP, X-linked inhibitor of apoptosis protein; zVAD, benzyloxycarbonyl-valine-alanine-aspartate-fluoromethylketone.

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