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Forum Review

Oxidative Stress and Growth-Regulating Intracellular Signaling Pathways in Cardiac Myocytes

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ABSTRACT

The toxic effects of oxidative stress on cells (including cardiac myocytes, the contractile cells of the heart) are well known. However, an increasing body of evidence has suggested that increased production of reactive oxygen species (ROS) promotes cardiac myocyte growth. Thus, ROS may be 'second messenger' molecules in their own right, and growth-promoting neurohumoral agonists might exert their effects by stimulating production of ROS. The authors review the principal growth-promoting intracellular signaling pathways that are activated by ROS in cardiac myocytes, namely the mitogen-activated protein kinase cascades (extracellular signal-regulated kinases 1/2, c-Jun N-terminal kinases, and p38-mitogen-activated protein kinases) and the phosphoinositide 3-kinase/protein kinase B (Akt) pathway. Possible mechanisms are discussed by which these pathways are activated by ROS, including the oxidation of active site cysteinyl residues of protein and lipid phosphatases with their consequent inactivation, the potential involvement of protein kinase C or the apoptosis signal-regulating kinase 1, and the current models for the activation of the guanine nucleotide binding protein Ras. *Antioxid. Redox Signal.* 8, 2111–2124.

INTRODUCTION

THE HEART AND ITS CONSTITUENT contractile cells (cardiac myocytes) possess the potential for adaptive growth. Because mammalian cardiac myocytes become terminally differentiated during the perinatal period and are incapable of undergoing complete cycles of cell division, growth is achieved by the expansion of pre-existing cells (*i.e.*, hypertrophy). Hypertrophy of the whole ventricle (sometimes known as remodeling) can occur clinically in response to increases in overall hemodynamic load caused by, for example, hypertension or valvular incompetence (106). Regional ventricular hypertrophy may be seen following a survivable myocardial infarction where myocyte loss occurs in the ischemic area with replacement by scar tissue. To compensate for the loss of contractile tissue, surviving myocytes undergo hypertrophic growth to maintain cardiac function as far as possible (106). A variety of monogenic mutations (commonly in myofibrillar proteins) also causes familial hypertrophic cardiomyopathies through unknown mechanisms. *In vivo* or *ex vivo*, cardiac myocyte hypertrophy is induced by vasoactive peptides [endothelin-1 (ET-1), angiotensin II (ANGII)] or α-adrenergic agonists, though several other hypertrophic agonists and interventions (*e.g.*, anabolic mechanotransduction, the process whereby cardiac myocyte growth occurs in response to increased mechanical strain) have been identified. A detailed description of cardiac myocyte hypertrophy is outside the scope of this article but can be found in recent reviews of the topic (55, 105, 106).

Through limiting their ability to divide, the terminally differentiated nature of cardiac myocytes renders the heart vulnerable to cellular stresses that promote myocyte death, be it by necrosis or apoptosis. One of the key stresses encountered by the heart is production of reactive oxygen species (ROS), which may contribute to remodeling and to other cardiac pathologies (57, 106). A variety of radical and nonradical forms of O_2 are produced *in vivo* as products of normal metabolic activity and enzymic action. ROS radicals include superoxide

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anion and hydroxyl radicals, whereas nonradical forms include H_2O_2. These are generated by the incomplete reduction of O_2 by successive addition of single electrons (57). Intracellularly, as much as 1–2% of the mitochondrial O_2 uptake is incompletely reduced and this proportion is increased by ischemia followed by reperfusion or, paradoxically, by ischemia alone (8, 128). This means that, at high workloads under normal *in vivo* conditions, the intracellular water space in the cardiac myocyte could accumulate ROS at a rate of about 1 μmol/ml per min. There are other sources of ROS. For example, NAD(P)H oxidases (NOXs) have recently received much attention as a source of superoxide anions in the cardiovascular system (88, 97). Singlet O_2, an excited, highly reactive state of molecular O_2, is formed by myeloperoxidases and other reactions (79, 140). ROS may combine with other molecules to form further reactive species (*e.g.*, superoxide anion and NO combine to form peroxynitrite). However, although there are abundant sources of ROS in cells, there are also efficient mechanisms (superoxide dismutases, catalase, peroxidases, etc.) that lead to their removal and thereby reduce the potential for cellular damage. Here, we review the principal intracellular signaling pathways that have been shown to be associated with hypertrophy and that are also modulated by ROS in cardiac myocytes, with particular emphasis on potential mechanisms of modulation.

INTRACELLULAR SIGNALING PATHWAYS ACTIVATED BY ROS

ROS as signaling molecules

Although severe oxidative stresses are cytotoxic to many cells [including cardiac myocytes (3, 47)], less severe stresses may be anabolic (85). There is now a fairly extensive literature showing that imposition of mild oxidative stress in the cardiac myocyte is hypertrophic (125). For example, in cultures of cardiac myocytes, direct pulsed imposition of moderate oxidative stress (H_2O_2) kills about half of the cells, but those that survive appear to display hypertrophic growth (28). Furthermore, as described in detail below, a number of hypertrophic stimuli increase ROS formation in cardiac myocytes. Indeed, the formation of ROS by such stimuli has been proposed to be a necessary process for the development of the hypertrophic response (125), and thus ROS serve as 'second messengers' in their own right (115, 117). This is a particularly attractive hypothesis since it could be argued that increases in O_2 consumption occurring during cardiac myocyte overload lead to an increased rate of production of ROS and thus promote hypertrophy.

However, there would seem to be a number of conceptual problems with the proposal that low levels of oxidative stress are anabolic. First, unlike other signaling intermediates (*e.g.*, second messengers such as cyclic AMP), ROS would seem inherently unlikely to possess the high level of specificity necessary for a signaling molecule. Second, for a signaling molecule to be anabolic at a low concentration also to be demonstrably cytotoxic at a higher concentration is unexpected [though it could be argued that it is not unknown for some physiologically important substances (*e.g.*, free long chain fatty acids) to behave in this manner]. Indeed, from a cellular perspective, using a molecule to promote growth that is cytotoxic at a higher concentration is potentially dangerous. Third, although ROS modulate a number of well-established signaling pathways in cardiac myocytes that are also activated by hypertrophic stimuli, activation is achieved only at the higher concentrations that are associated with cell death (42, 47). Finally, there are few clearly established mechanisms by which ROS activate such growth-promoting signaling pathways. Many of the proposed mechanisms involve oxidation of the sulphydryl groups in cysteinyl residues. There are several potential oxidation states of sulphydryl groups: -SH itself, disulphide bond formation, -SOH, $-SO_2H$, and $-SO_3H$, and the end result of oxidation will depend on redox potentials and reactant concentrations. Although most of these sulphydryl oxidations are biologically reversible, the oxidation to sulphonic acid ($-SO_3H$) probably is not (116, 149). Thus, ROS may not possess two of the features for a signaling molecule, namely specificity and reversibility. Nevertheless, there is undoubted evidence that ROS do modulate intracellular signaling pathways. The important question is whether this is a physiological, rather than a pathological event.

Reversible protein phosphorylation and dephosphorylation

Reversible covalent modification of proteins represents a common means of regulating their biological activity. One of the most frequent modifications in intracellular signaling is reversible phosphorylation and dephosphorylation by protein kinases and protein phosphatases, respectively. Application of such phosphorylation/dephosphorylation cycles to successive protein kinases themselves generates a number of different protein kinase cascades. The principal cascades that have been associated with cardiac myocyte hypertrophy include a number of mitogen-activated protein kinase (MAPK) cascades and protein kinase B (PKB, also known as Akt), all of which are activated by ROS.

Mitogen-activated protein kinase cascades

All well-established MAPKs (29) contain a Thr-Xaa-Tyr sequence in their activation loops, and phosphorylation of both the Tyr- and Thr-residues is required for activation (Fig. 1). For the extracellular signal-regulated kinases (ERKs), Xaa is most commonly Glu-. For the c-Jun N-terminal kinases (JNKs), Xaa- is Pro-, whereas it is Gly- for the p38-MAPKs. MAPKs are phosphorylated and activated by MAPK kinases (MKKs), which are in turn phosphorylated and activated by MKK kinases (MKKKs) and these MKKK–MKK–MAPK 'cassettes' constitute the various three-tiered MAPK cascades. Activation of MAPKs is reversed by their dephosphorylation by Ser-/Thr-, Tyr-, or dual-specificity protein phosphatases.

The ERK cascades

Though the nomenclature is confused and confusing, as many as eight 'ERKs' (ERKs 1–8) have been identified (14, 29). However, ERK6 is not an ERK by the phosphorylation

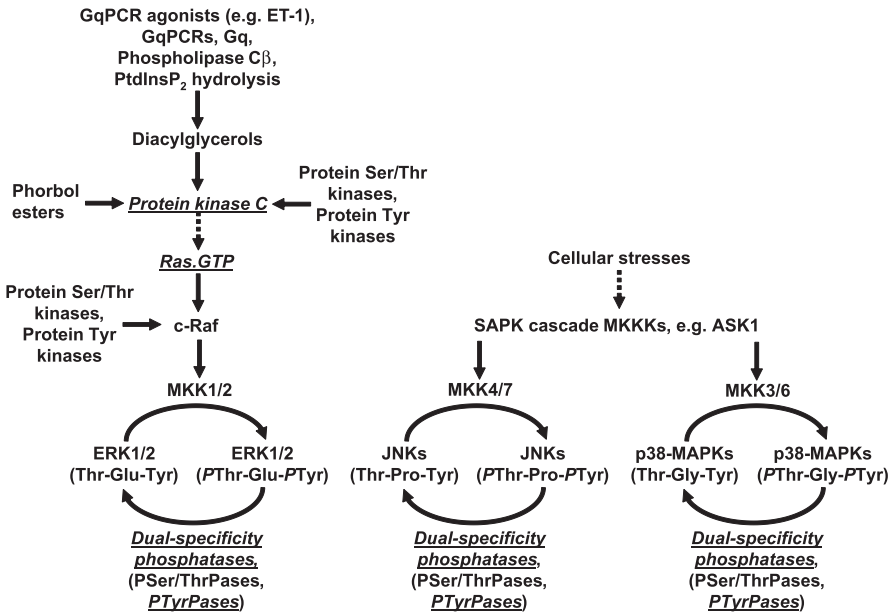

FIG. 1. Activation of mitogen-activated protein kinase (MAPK) cascades. The extracellular signal-regulated kinase 1/2 (ERK1/2) cascade is the archetype. Proteins potentially susceptible to modulation by reactive oxygen species (ROS) are in *italics* and underlined. Further details in the text under the appropriate sections. Activation of the guanine nucleotide binding protein Ras (H-, K- and N-Ras isoforms) by stimulation of exchange of GTP with Ras-bound GDP increases Ras.GTP loading. The Raf isoforms (c-Raf is shown) have a high affinity for Ras.GTP and translocate to the membrane. In the case of c-Raf, phosphorylations and probably dephosphorylations occur and c-Raf becomes fully activated. Raf then phosphorylates two Ser-residues on the MAPK kinases, MKK1 and MKK2, activating them. MKK1/MKK2 then phosphorylate ERK1 and ERK2 on a Tyr-residue and a Thr-residue, activating them in turn. Activation of c-Raf is reversed by Ras-mediated hydrolysis of the bound GTP and presumably by changes in its phosphorylation state. MKK1/2 are dephosphorylated by protein Ser/Thr phosphatases (PSer/ThrPases). ERK1/2 are dephosphorylated on both Tyr- and Thr-residues by the Cys-dependent dual-specificity protein phosphatases. There are at least 13 of these with differential specificity towards the various MAPKs, and they are either constitutively or inducibly expressed (52). Alternatively, protein Ser/Thr phosphatases or Cys-dependent protein Tyr phosphatases (PTyrPases) dephosphorylate their respective sites individually. The mechanisms of activation of the ERK1/2 cascade by Gq protein-coupled receptor (GqPCR) agonists such as endothelin-1 (ET-1) are incompletely understood. These agonists (or phorbol esters) activate the diacylglycerol-sensitive protein kinase C isoforms and this probably leads to activation of Ras, though the nature of the connections is unclear (*dashed arrow*). The stress-activated MAPKs (or SAPKs), which include the c-Jun N-terminal kinases (JNKs) and p38-MAPKs, are activated by protein kinases cascades that are analogous in terms of their organization to the ERK1/2 cascade (*i.e.*, an MKKK phosphorylates and activates an MKK which then phosphorylates and activates a MAPK). However, the proportional contributions of the various known MKKKs, such as apoptosis signal-regulating kinase 1 (ASK1), are unclear and precisely how the various diverse stresses activate these MKKKs is unclear (*dashed arrow*). Furthermore, activation of these cascades is reversed by dual-specificity protein phosphatases, protein Ser/Thr phosphatases and/or protein Tyr phosphatases. The ERK5 cascade, which is also activated by cellular stresses, is not shown. The kinase immediately upstream of ERK5 is MKK5.

motif criterion (it was simultaneously termed p38-MAPKγ), the phosphorylation site in ERK3 is atypical, and that in ERK4 has not been characterized. ERK7 and ERK8 have not been extensively investigated, but are classified as ERKs on the basis of their phosphorylation motifs. The best characterized ERKs are ERK1, ERK2, and ERK5 (also known as 'big' MAP kinase, BMK1). Although ERK1 and ERK2 are products of separate genes, they appear to be always activated in concert and there is little evidence of any differential function. ERK1/2 are phosphorylated and activated by the upstream kinases MKK1 and MKK2, which are in turn phosphorylated and activated by the Raf family of MKKKs (A-Raf, B-Raf, and c-Raf) (Fig. 1). ERK5 is phosphorylated by MKK5, but the upstream MKKKs are not yet well defined.

ERK1/2 are classically activated by peptide growth factors that signal through receptor protein Tyr- kinases (RPTKs) (126) and, in cardiac myocytes, they are activated by epidermal growth factor (EGF), fibroblast growth factors, and platelet-derived growth factor, but they are not significantly activated by insulin or insulin-like growth factor 1 (IGF1) (37). In cardiac myocytes, ERK1/2 are also particularly strongly activated by neurohumoral hypertrophic stimuli such as ET-1 that act through the Gq subclass of G protein-coupled receptors (GPCRs) (16, 17). For GqPCR agonists, the diacylglycerol-activated isoforms of the phospholipid-dependent protein kinase, protein kinase C (PKC), participate in activation of ERK1/2, and hence ERK1/2 are also strongly activated by suitable growth-promoting phorbol esters [*e.g.*, phorbol 12-myristate 13-acetate (PMA)] in cardiac systems. Activation of ERK1/2 is generally associated with cell growth and survival, and studies with transgenic mice have shown that selective activation of the ERK1/2 cascade in the myocardium induces an adaptive cardiac hypertrophy (25). Further evidence linking ERK1/2 with hypertrophy has been reviewed (26).

ERK5 (14, 63) is expressed in neonatal rat cardiac myocytes (77) but little is known about its regulation in these cells in general or by ROS in particular. In other cells (vascular smooth muscle cells), it is thought to be a 'redox-sensitive' MAPK and is activated by ROS (1). Although reportedly activated by the gp130-linked cytokine cardiotrophin-1 in cardiac myocytes and to be pro-hypertrophic (102, 135),

ERK5 is not detectably activated by ET-1 but is activated by the stress of hyperosmotic shock induced by 0.5 *M* sorbitol (77), suggesting that it is effectively a 'stress-activated' MAPK (see below).

The 'stress-activated protein kinases' of the MAPK superfamily

The JNKs and p38-MAPKs are potently activated by cellular stresses, and are hence also known as the 'stress-activated protein kinases' (SAPKs) to distinguish them from ERK1 and ERK2 (7, 45, 133). In *Homo sapiens*, there are three *JNK* genes that give rise to multiple alternatively-spliced transcripts (60). In non-neuronal tissues, transcripts encoding proteins of approximately 46 and 54 kDa originate from both the *JNK1* and *JNK2* genes. [It is possibly a fairly common misconception that the *JNK1* gene encodes the 46 kDa JNK species and the *JNK2* gene encodes the 54 kDa JNK species. However, as Gupta *et al*. (60) demonstrate, this is not the case.] There are four *p38-MAPK* genes (including ERK6), transcripts for two of which are alternatively spliced (82, 91, 123). There is still discussion about the diversity of expression of p38-MAPK isoforms in heart (48, 92), though work on this system has probably concentrated on those that are inhibited by SB203580 (*i.e.,* the α and β isoforms) (82). JNKs are phosphorylated and activated by the upstream MKKs 4 and 7, whereas p38-MAPKs are activated by MKKs 3 and 6 (Fig. 1). However, the identities of the MKKKs for these are unclear with a number being identified that may operate under different conditions of cellular stress.

In cardiac myocytes, SAPKs are phosphorylated and strongly activated by cellular stresses (*e.g.*, hyperosmotic shock) but they are only poorly activated by PMA (18, 41). However, somewhat surprisingly, they are significantly activated by GqPCR agonists such as ET-1 and the α-adrenergic agonist, phenylephrine (18, 41). In perfused hearts *ex vivo*, the SAPKs are strongly activated by ischemia–reperfusion (15), and activation by phenylephrine is also detectable (90). Whilst evidence has been presented that certain members of the SAPKs are hypertrophic (55, 105, 133) or mediate cell survival (particularly if activated at certain critical stages) (5, 51, 74), our view is that the evidence is less clear than for ERK1 and ERK2. Indeed, the problems (myocyte death) associated with experimental ischemia–reperfusion might be related to activation of SAPKs because a p38-MAPKα/β inhibitor (SB203580) reduces the extent of cardiac myocyte apoptosis and infarct size (93, 138). The predominating opinion from a multitude of studies in noncardiac and cardiac systems is that the SAPKs preside over cell death (7).

MAPKs and ROS in the myocardium

ERK1/2 are activated by ROS in cardiac myocytes and perfused hearts (3, 40, 42, 84, 120). In our hands, direct imposition of oxidative stress (0.1 m*M* H_2O_2, 30 min) activates ERK1/2 in neonatal rat myocytes to a similar extent as PMA (42), which activates ERK1/2 maximally in this system. However, in perfused adult rat hearts, the relative activation is much less (40). Thus, it may be that oxidative stress is less effective in stimulating the ERK1/2 cascade in the adult situation and this may influence the ability of oxidative stress to promote myocyte growth in this situation. In both neonatal rat cardiac myocytes and in perfused adult rat hearts, the JNKs and p38-MAPKs are activated by H_2O_2 (40, 42, 84) and ROS may contribute towards the activation of JNKs and p38-MAPKs by ischemia–reperfusion in isolated hearts (40) or the hypoxia-induced activation of JNKs in isolated cardiac myocytes (87). *In vivo*, imposition of oxidative stress in the heart can be achieved in transgenic mice by cardiomyocytic expression of dominant–negative (inhibitory) thioredoxin (153). This leads to activation of ERK1/2 (and induction of cardiac hypertrophy) but, somewhat surprisingly, SAPKs are unaffected. It is worth emphasizing that H_2O_2 increases MAPK activities (40, 41). Although phosphorylation of MAPKs as measured with phospho-specific antibodies is quite reasonably assumed to equate to their activation, this does depend on the assumption that such antibodies recognise only the dually-phosphorylated species.

The protein kinase B (PKB) pathway

PKB (also known as Akt) (22, 23, 143, 150) is a key regulator of cell growth and survival in the heart (94) and other tissues. It is classically activated by RPTKs (archetypically by the insulin and IGF1 receptors) through phosphoinositide 3-kinase (PI3K) (144) and 3-phosphoinositide-dependent kinase 1 (PDK1) (Fig. 2). This involves the phosphorylation of the membrane phospholipid phosphatidylinositol 4,5-*bis*-phosphate [PtdIns(4,5)P_2] to PtdIns(3,4,5)P_3 by PI3K, which in turn leads to the activation of PDK1 and PKB. As has been recently elucidated (see Fig. 2), the situation is somewhat more complex than this (10), though the basic scheme still holds true. Hydrolysis of PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2 by the lipid phosphatase PTEN terminates PtdIns(3,4,5)P_3 signaling (144). Alternatively, hydrolysis of the 5-phosphate by a second group of lipid phosphatases (SHIPs) is also possible, though PtdIns(3,4)P_2 is probably also a signaling molecule in its own right.

In neonatal rat cardiac myocytes, PKB phosphorylation (activation) is induced by insulin and other RPTK agonists but phosphorylation in response to ET-1, α-adrenergic agonists or PMA is much less (37, 110). However, somewhat surprisingly, concentrations of H_2O_2 that induce myocyte death (>0.5 m*M*) stimulate phosphorylation and activation of PKB to a similar extent as insulin and, as with insulin, activation of PKB by H_2O_2 is dependent on PI3K activity (110). H_2O_2 also modulates upstream and downstream events associated with activation of the PKB pathway [Tyr-phosphorylation of the p85 regulatory subunits of PI3K, increased PI3K activity, phosphorylation of p70 ribosomal subunit S6 kinase 1 (142)]. Unlike the situation with insulin, however, PKB activation by H_2O_2 is not associated with any increase in initial rates of global protein synthesis or phosphorylation of translation initiation regulator 4E-BP1 (110). In fact, protein synthesis rates and 4E-BP1 phosphorylation are reduced by H_2O_2. This is probably attributable to supervening effects of H_2O_2 that counteract the activation of PKB by unknown mechanisms. Because the protein phosphatase 1/2A inhibitor okadaic acid (46) inhibits the H_2O_2-induced decreases in 4E-BP1 phosphorylation, one possibility is that an okadaic acid-sensitive protein phosphatase activity is in some way induced (110).

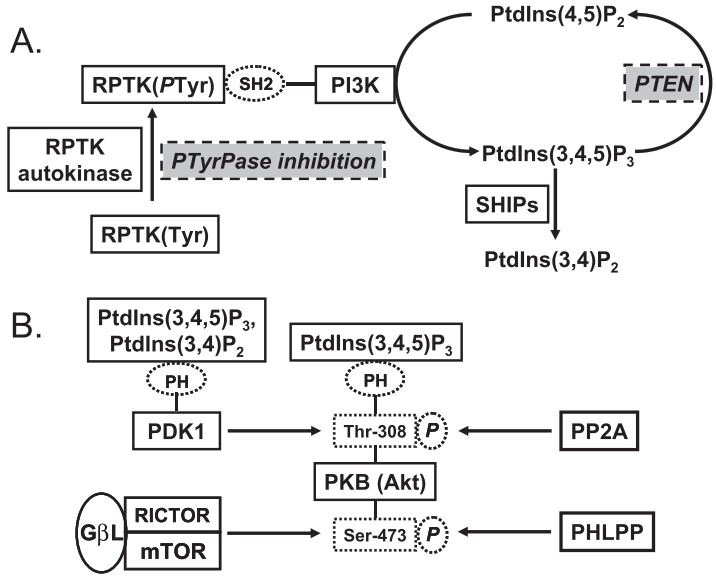

FIG. 2. Phosphatidylinositol 3,4.5-trisphosphate [PtdIns(3,4,5)P_3] turnover and activation of PKB/Akt. (**A**) Proteins potentially susceptible to inhibition by reactive oxygen species are in *italics* and enclosed in *long-dashed rectangles filled with shading*. Activation of receptor protein tyrosine kinases (RPTKs) by their individual extracellular ligands leads to their autophosphorylation on specific Tyr-residues Alternatively, inhibition of cysteine-dependent protein Tyr-phosphatases (PTyrPases) by oxidative stress, when coupled with tonic low level RPTK autokinase activity, increases Tyr-phosphorylation of RPTKs. In either situation, such increased Tyr-phosphorylation promotes binding of specific Src homology 2 (SH2) domain-containing proteins such as phosphoinositide 3-kinase (PI3K) to these *P*Tyr-residues. In the case of the PI3K, it is the regulatory subunit of the regulatory subunit–catalytic subunit dimer that binds, and this event places the PI3K catalytic subunit in the plane of the membrane where it phosphorylates PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3. Some isoforms of PI3K are activated by GPCRs rather than RPTKs. Formation of PtdIns(3,4,5)P_3 by PI3K is opposed by the Cys-dependent lipid phosphatase, PTEN (phosphatase and tensin homologue deleted on chromosome 10). Alternatively, PtdIns(3,4,5)P_3 is hydrolyzed to PtdIns(3,4)P_2 by SH2 domain-containing lipid phosphatases such as the SHIPs (SH2-containing inositol 5'-phosphatases). (**B**) In the unstimulated state, a proportion of the 3-phosphoinoitide-dependent kinase 1 (PDK1) is bound to the membrane through its phospholipid-binding pleckstrin-homology (PH) domain by the basal levels of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 present. When PI3K is activated, PtdIns(3,4,5)P_3 levels in the plane of the membrane increase and protein kinase B (PKB) translocates to this locale, again binding through its own PH domain (which has a lower affinity for 3-phosphoinositides than PDK1). The juxtapositioning of PDK1 and PKB allows PDK1 to phosphorylate Thr^{308} in PKB. In addition to this site, Ser^{473} in PKB needs to be phosphorylated in order for the enzyme to be maximally activated. This is carried out by a poorly understood mechanism involving the mammalian target-of–rapamycin (mTOR) complexed with two other proteins, GβL and RICTOR (10). This form of mTOR is not sensitive to inhibition by rapamycin. Dephosphorylation of Thr^{308} is probably catalyzed by the protein phosphatase PP2A, whereas dephosphorylation of Ser^{473} is catalyzed by the PH domain-containing phosphatase PHLPP (PH domain leucine-rich repeat protein phosphatase) (10).

GqPCR agonists and ROS production in the myocardium

There is evidence that hypertrophic GqPCR agonists (ET-1, ANGII, α-adrenergic agonists) stimulate ROS production in the heart (4, 31, 70, 99, 131, 137, 151), with ANGII and possibly the other agonists stimulating superoxide anion production by increasing NOX activity (97, 137). The same is true for mechanical deformation (strain) of the cardiac myocyte (111) and, though it is not certain how myocytes detect strain, autocrine or paracrine release of GqPCR agonists may be involved (121, 132). An obvious question is whether ROS are an obligate intermediate in the activation of MAPKs by GqPCR agonists or strain. This may seem inherently unlikely for the ERK1/2 cascade where an alternative well-established mechanism involving the guanine nucleotide binding protein Ras is operative, though it may be inappropriate to exclude a role for ROS altogether. Indeed, small molecule antioxidants decrease stimulation of ERK1/2 phosphorylation by GqPCR agonists or strain (137, 152), as (somewhat surprisingly) does extracellular catalase (137) implying participation of *extracellular* ROS. For the SAPKs, the question of the role of ROS as signaling intermediates is perhaps more pertinent since there is no well-established pathway for their activation by GqPCR agonists. Studies of SAPK activation by GqPCR agonists in noncardiomyocytic cells lends some support to these views (30, 86, 141) and, furthermore, the activation of JNKs by strain in cardiac myocytes is reduced by antioxidants (111). *In vivo*, administration of ANGII or phenylephrine causes an elevation of blood pressure and antioxidant-sensitive activation of MAPKs (154). This suggests that ROS are involved in activation of MAPKs. However, because the elevation of blood pressure is insensitive to antioxidants, the production of ROS could result from a direct effect of the GqPCR agonists on the heart or could be related to the increases in blood pressure (154).

How might GqPCR agonists increase myocardial ROS production? Two mechanisms come immediately to mind. GqPCR agonists such as ET-1, ANGII and α-adrenergic agonists are positively inotropic and thus increase O_2 uptake because of a requirement for an increased rate of oxidative phosphorylation. Assuming that the proportion of O_2 that is incompletely reduced does not alter, this will potentially lead to increased rates of ROS formation. Alternatively, there could be a role for NOXs. NOX2, which is present in the myocardium, is activated by the GTP-ligated (activated) form of the small guanine nucleotide binding protein Rac1 (88, 97). ET-1 or phenylephrine increases Rac1 GTP-loading in cardiac myocytes (43), and thus this could lead to an increase in NOX2 activity.

ACTIVATING THE OXIDATIVE STRESS-RESPONSIVE SIGNALING PATHWAYS

It is unlikely that there are specific 'receptors' for oxidative stress in the same sense as in hormonal signaling. There is increasing evidence that post-translational reversible modification of cysteinyl sulphydryl groups in proteins represents an important physiological mechanism through which ROS can regulate cell signaling events (103). Modifications include reversible oxidation of Cys- to its sulphenic and sulphinic acid derivatives (and possibly irreversible oxidation to Cys-sulphonic acid), Cys-glutathionylation, cysteinylation, and cysteaminylation, and Cys-nitrosylation (103). In general terms, the four groups of signaling proteins most studied in relation to oxidative stress signaling are the sulphydryl-dependent phosphatases, the PKC family, the MKKK known as apoptosis signal-regulating kinase 1 (ASK1) and the Ras guanine nucleotide binding proteins. It should be noted that these mechanisms may not be independent, for example, inhibition of sulphydryl-dependent protein Tyr-phosphatases could be responsible for an increase in Tyr-phosphorylation of PKC. The question is whether there is any evidence that ROS influence signaling events in cardiac myocytes by these mechanisms?

Inhibition of phosphatases by ROS

Given that regulatory protein phosphorylation and dephosphorylation are reversible, the phosphorylation state of a protein can be increased by the activation of a protein kinase, by the inhibition of a protein phosphatase, or by both of these operating in conjunction (Figs. 1 and 2). A similar consideration applies to regulatory molecules that are synthesised and degraded by kinase and phosphatase steps [*e.g.,* PtdIns $(3,4,5)P_3$]. It should be noted that increases in phosphorylation state in the face of phosphatase inhibition will be dependent on a tonic level of kinase activity. Several groups of phosphatases (various protein Tyr-phosphatases, dual-specificity protein phosphatases, PTEN) contain at least one 'reactive' Cys-residue in conserved motifs in their active sites (9, 52, 122). This Cys-residue is responsible for hydrolytic, nucleophilic attack, and oxidation of this residue is inhibitory (9, 122). The question is whether these oxidations are reversible, an important consideration in physiological signaling. Complete oxidation of an active site Cys-residue to Cys-sulphonic acid is probably a biologically irreversible process whilst reversible incomplete oxidation of Cys-dependent phosphatases may be a more important mechanism from a biological standpoint (9, 115, 117, 122). However, both irreversible and reversible oxidation could result in increased substrate phosphorylation. RPTKs regulate signaling pathways by their degree of autophosphorylation on specific Tyr-residues. Thus, for example, H_2O_2 increases Tyr-phosphorylation of the EGF RPTK in vascular smooth muscle cells [H_2O_2 stimulates growth and division of these cells (59)] and activates the ERK1/2 cascade (112). One possible explanation is that inhibition of protein Tyr-phosphatases, in the face of tonic low-level autophosphorylation, increases receptor phosphorylation. A similar mechanism might account for the insulin-mimetic effect of peroxyvanadate stress through the insulin RPTK (13). In cardiac fibroblasts, ET-1 stimulates ROS generation and EGF receptor phosphorylation by oxidation and inhibition of the protein Tyr-phosphatase, SHP-2 (27). In cardiac myocytes, H_2O_2 causes a general increase in Tyr-phosphorylation (20), consistent with the concept that oxidative stress promotes general inhibition of protein Tyr-phosphatases. Equally, inhibition of PTEN should promote the accumulation of PtdIns$(3,4,5)P_3$, and this may account for the observed phosphorylation and activation of PKB by H_2O_2 (110).

One protein phosphatase that has been directly implicated in cardiac myocyte growth is the Ca^{2+}/calmodulin-dependent protein Ser-/Thr- phosphatase PP2B or calcineurin (147). Unlike the phosphatases discussed above, calcineurin does not possess an active site cysteine(s). From the point of view of Ca^{2+} (*i.e.*, physiological activation of calcineurin), oxidative stress causes intracellular overload of Ca^{2+} and dysregulation of Ca^{2+} movements in cardiac myocytes (58, 78). However, from the point of view of direct effects of ROS on signaling proteins (*i.e.*, the topic of this section), this phosphatase appears to be inhibited by ROS (129) that interact with the dinuclear Fe^{II}-Zn^{II} active site (21). Overall, therefore, calcineurin seems to be an unlikely candidate to mediate any growth-promoting effects of ROS.

The situation with respect to the effects of ROS on other protein Ser/Thr phosphatases is unclear. In relation to the PKB and MAPK cascades, this is an important consideration (recall that phosphorylation of both the Thr-residue and the Tyr-residue in the MAPK activation loop is necessary for activation). We have already mentioned that H_2O_2-induced activation of a protein phosphatase that is sensitive to okadaic acid [a potent inhibitor of protein Ser/Thr phosphatase 2A (46)] may account for the H_2O_2-induced reduction of 4E-BP1 phosphorylation (110). However, activation of such a phosphatase should inhibit MAPK activation. In other systems, the situation is unclear with H_2O_2 reported to inhibit (54, 104, 113), have relatively little effect (129), or activate (35) protein Ser/Thr phosphatases such as PP1 and PP2A.

Apoptosis signal-regulating kinase 1 (Ask1)

ASK1 (also known as MKKK5) is a redox status-sensitive MKKK for the JNK and p38-MAPK cascades (134). Given that some investigators have established a role for ROS, JNKs, and p38-MAPKs in cardiac myocyte hypertrophy (133), there is some basis for a belief that ASK1 may be involved. ASK1 is present in both the cytoplasm and mitochondria, but the cytoplasmic form is the better characterized. In its inactive state, ASK1 is associated with reduced thioredoxin and is phosphorylated on Ser^{967}. Phospho-Ser^{967} is recognized by 14–3-3 proteins, a family of proteins that can bind to phospho-Ser/phospho-Thr residues. In the presence of ROS or mixed species such as peroxynitrite, the thioredoxin thiol groups become oxidized and thioredoxin dissociates from ASK1. This is accompanied by dephosphorylation of phospho-Ser^{967}, dissociation from 14–3-3 proteins, ASK1 homodimerization, and its autophosphorylation on Ser^{845}. Tumor necrosis factor receptor associated factor 2 dimers [$(TRAF2)_2$] then bind to the ASK1(phospho-Ser^{845}) dimers and this complex phosphorylates and activates the MKKs for the JNK and p38-MAPK cascades (MKK4 and/or MKK7,

and MKK3 and/or MKK6, respectively). It is also thought that redox proteins such as glutaredoxin or glutathione *S*-transferases μ or π can act in a manner analogous to thioredoxin to inhibit ASK1. In addition, glutathione *S*-transferases may bind to and inhibit the JNKs, an association that is also redox status sensitive.

Regulation of ASK1 activity has not been examined in great detail in the heart, and the majority of studies so far have used either transfection of isolated myocytes or mice in which the ASK1 gene has been deleted. In mice infused with ANGII, cardiac production of ROS is increased, resulting in activation of ASK1, JNKs, and p38-MAPKs (73). These changes are inhibited by the antioxidant tempol or by targeted gene deletion of ASK1 (73). Similarly, ischemia or ischemia–reperfusion activates ASK1 (and p38-MAPK) (145). There is thus somewhat indirect evidence that, as expected, redox status controls ASK1 activity in cardiac tissue, but what is not clear is whether the response is related to an adaptive hypertrophy or a cardiomyopathy. It is interesting to note in this regard that cardiospecific expression of dominant–negative (inhibitory) thioredoxin in mice *in vivo* increases heart size whereas overexpression of wild-type thioredoxin suppresses hypertrophy induced by pressure overload (153).

Protein kinase C isoforms

The PKC family are phospholipid-dependent kinases that translate signaling events in the plasma membrane further downstream (100, 101, 107). The 'genuine' PKCs comprise a family of kinases transcribed from nine genes in mice or *Homo sapiens* and they fall into three subfamilies. Classical PKCs (cPKCs) are regulated by Ca^{2+} and diacylglycerol, novel PKCs (nPKCs) are also regulated by diacylglycerol, but the regulation of atypical PKCs is not well understood. Receptor-mediated hydrolysis of PtdIns(4,5)P_2 produces inositol 3,4,5-trisphosphate, which regulates intracellular Ca^{2+} movements, and diacylglycerol. Following its formation, diacygglycerol remains in the plane of the membrane and diacylglycerol-activated isoforms of PKC (predominantly present in the soluble phase of unstimulated cells) migrate to the particulate (membrane) fraction. This behavior is frequently used as a surrogate index of activation of cPKCs and nPKCs because assay of PKC activity *per se* is challenging for a variety of technical reasons. Phorbol esters such as PMA act as membranotropic diacylglycerol analogues and can help to implicate PKC-dependent signaling in biological processes. In addition to this well-defined format of activation, PKCs are phosphorylated on Ser-/Thr- and Tyr residues that may facilitate PKC activation (*i.e.*, they are required for PKC to be activated) or may directly regulate PKC activity. Less is known about regulation of PKCs by phosphorylation and dephosphorylation than about their regulation by diacylglycerols.

As described above, ERK1/2 are activated by H_2O_2 in cardiac myocytes. Though the evidence is not definitive, this activation may involve c/nPKCs since prolonged treatment with PMA [which downregulates diacylglycerol-responsive PKCs in cardiac myocytes (39)] or acute exposure to GF109203X (an allegedly 'selective' PKC inhibitor) suppresses activation of ERK1/2 by H_2O_2 (42). The mechanism by which ROS regulate PKC activity is unclear. In COS-7 cells, transfected 'tagged' PKCs are activated by H_2O_2 (as shown by immunoprecipitation and enzyme activity measurements), but this involves Tyr-phosphorylation of the PKC catalytic domains and the PKC activity is apparently independent of lipid cofactors (80). Tyr-phosphorylation of nPKCδ in its catalytic, regulatory, and hinge domain has been studied most extensively (81, 130). In neonatal rat cardiac myocytes, high concentrations of H_2O_2 (5 m*M*) promote phosphorylation of Tyr^{311} which lies in the hinge region of nPKCδ (but may also promote phosphorylation of other Tyr-residues for which specific antibodies were not available) (119). This is associated with *release* of the nPKCδ fraction that is bound to the particulate fraction in the basal state into the soluble fraction [in cardiac myocytes, as much as 50% of nPKCδ may be in the particulate phase (39, 119)] and increased lipid-independent nPKCδ catalytic activity.

How might Tyr-phosphorylation of nPKCδ be caused by H_2O_2? It could simply be through protein Tyr-phosphatase inhibition, as discussed above. However, this is not the favored explanation. The Src family of nonreceptor protein Tyr-kinases is important generally in cellular regulation (118). In its inactive state, c-Src is phosphorylated on Tyr^{527} (numbering is for chicken c-Src), a phosphorylation catalyzed by C-terminal c-Src kinase, CSK, and the related CSK-homologous kinase (33, 118). This phosphorylation is inhibitory because it maintains c-Src in a conformationally 'closed' state. In addition, there is an activating autophosphorylation site at c-Src(Tyr^{416}) in the c-Src catalytic domain. The c-Src(phospho-Tyr^{527}) site needs to be dephosphorylated to 'relax' the c-Src conformation before c-Src(Tyr^{416}) phosphorylation can occur. In cardiac myocytes, ROS induce the activating phosphorylation of Tyr^{416} (in c-Src itself) and phosphorylation of the equivalent Tyr-residue in other Src family kinases (119). These kinases are responsible for the ROS-induced Tyr-phosphorylation of nPKCδ as shown by experiments involving their selective inhibition (119). Although Rybin *et al*. (119) showed that H_2O_2 increased Tyr^{416} phosphorylation, they do not seem to have studied the phosphorylation of Tyr^{527}. It is somewhat difficult to rationalize activation of c-Src by dephosphorylation of Tyr^{527} and concurrent phosphorylation of Tyr^{416} in terms of global inhibition of protein Tyr-phosphatases by ROS (see above) because the inhibitory Tyr^{527} needs to be dephosphorylated by an active protein Tyr-phosphatase before phosphorylation of Tyr^{416} could be increased by autophosphorylation (possibly coupled with inhibition of protein Tyr-phosphatases).

It is worth noting that the concentrations of H_2O_2 used by Rybin *et al*. (119) to promote Tyr-phosphorylation of nPKCδ are considerably higher than those reported to be necessary for maximal activation of ERK1/2 (42). Although this may be attributable to different conditions for cell culture and/or rates of H_2O_2 breakdown, we have been unable to detect significant phosphorylation of nPKCδ at concentrations of H_2O_2 at which ERK1/2 are maximally activated (Clerk and Sugden, unpublished data), raising the question of whether nPKCδ could indeed promote ERK1/2 activation in this context. In addition to Tyr-phosphorylation, ROS-mediated activation of nPKCδ may involve direct modification of cysteinyl-residues in nPKCδ itself (34), though this does not appear to have been explored in the cardiac myocyte.

The situation with respect to the involvement of nPKCδ in hypertrophic growth is rather more complex. First, signifi-

cantly higher concentrations of ET-1 or phenylephrine are required to induce its translocation to the particulate fraction than are required for nPKCϵ (38). The interpretation is that nPKCϵ activation by these agonists is favored over activation of nPKCδ. Second, whereas nPKCϵ signals to the ERK1/2 cascade in cardiac myocytes (64), nPKCδ signals to the JNK and p38-MAPK cascades (64, 124). Third, rather than promoting cell growth, nPKCδ is said to be a 'proapoptotic' PKC isoform (24, 61, 75), and its translocation to the mitochondria following ischemia/reperfusion may induce cell death in the heart by stimulating the mitochondrial pathway of apoptosis (98). The scheme in neuronal cells is somewhat different where ischemia or ROS cause a caspase-3-dependent cleavage of nPKCδ in the hinge region (where Tyr^{311} is located) to free the C-terminal catalytic domain fragment of ~40 kDa (75, 76, 109, 114). This cleavage is apparently enhanced by the ROS-stimulated phosphorylation of Tyr^{311}. Cleavage of PKCs in the hinge region frees the C-terminal catalytic domain from the regulatory domain and the produces a constitutively active 'unregulated' species. However, to our knowledge, no such cleavage of nPKCδ has yet been reported in cardiac myocytes.

Oxidative activation of Ras in the heart

A number of signaling pathways are dependent on the activation of small guanine nucleotide binding proteins (136) for their own activation, and such proteins have been implicated in cardiac hypertrophy (44). Small guanine nucleotide binding proteins constitute a large superfamily of which H-Ras was the first to be characterized, with the K- and N-homologues being identified subsequently. Any of these activates the three Raf isoforms and thence the ERK1/2 cascade, and Ras may also be involved in the activation of PI3K and the PKB pathway. Partitioning of H-Ras and K-Ras to the cytoplasmic face of the plasma membrane is essential to their signaling functions and this is achieved by as many as four successive post-translation modification steps, described in detail in Fig. 3 (12, 95, 136, 148). Irreversible farnesylation in the C-terminal region by protein farnesyltransferases is followed by cleavage of the terminal tripeptide by the RCE1 peptidase, with ensuing C-terminal carboxyl methylation. Interestingly, postnatal cardio-specific deletion of the RCE1 gene in mice results in the development of a dilated cardiomyopathy (11). For H-Ras, membrane localization is possibly reinforced by reversible *S*-acylation with palmitoyl-CoA-derived palmitate. Ras.GDP is the biologically inactive Ras species and it is activated by the regulated exchange of GDP for GTP (Fig. 4). Agonist-stimulation activates GDP/GTP exchange on Ras (probably enhanced by regulated guanine nucleotide exchange factors) and GTP-binding induces a conformational change that allows the consequent binding of Raf to the membrane-localized Ras.GTP. Localization of Raf at the membrane allows further modifications to take place, resulting in its full activation and the subsequent activation of the ERK1/2 cascade (146). Subsequently, the innate GTPase activity of Ras (probably increased by GTPase-activating proteins) returns Ras to the biologically inactive GDP-ligated state. Powerful activation of this signaling pathway is seen with GqPCR agonists (*e.g.*, ET-1) or PMA in cardiac myocytes (16, 17, 19, 32).

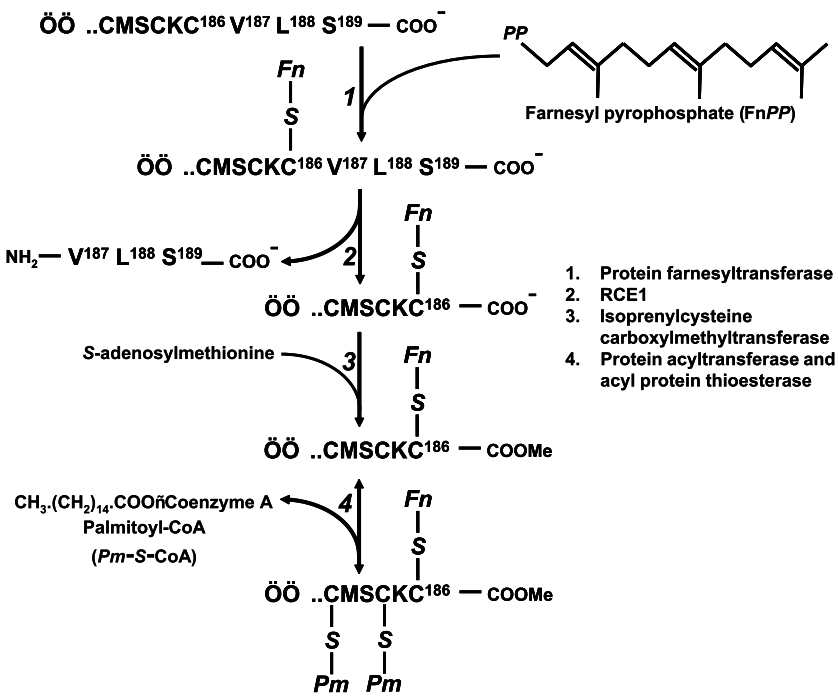

FIG. 3. Maturation of H-Ras. H-Ras is synthesized as a 189 residue precursor. It is then irreversibly *S*-farnesylated on Cys^{186} by cytoplasmic protein farnesyltransferases with farnesyl pyrophosphate (FnPP) as farnesyl donor, and inorganic pyrophosphate is released. Farnesylated H-Ras travels to the endoplasmic reticulum where the three terminal amino acids are cleaved by the RCE1 prenylprotein peptidases. The now C-terminal farnesylated Cys^{186} is irreversibly carboxyl methylated by an isoprenylcysteine carboxylmethyltransferase. *S*-adenosylmethionine is the methyl donor in the reaction, and *S*-adenosylhomocysteine is formed. Processed H-Ras is then localized probably to specific regions on the cytoplasmic face of the plasma membrane. Reversible *S*-acylation (palmitoylation, Pm) of cysteinyl residues in the C-terminal hexapeptide region with palmitoyl-CoA as donor probably participates in this. The acylation is catalyzed by a protein acyltransferase, whereas the deacylation involves an acyl protein thioesterase. C, cysteine; K, lysine; L, leucine; M, methionine; S, serine; V, valine.

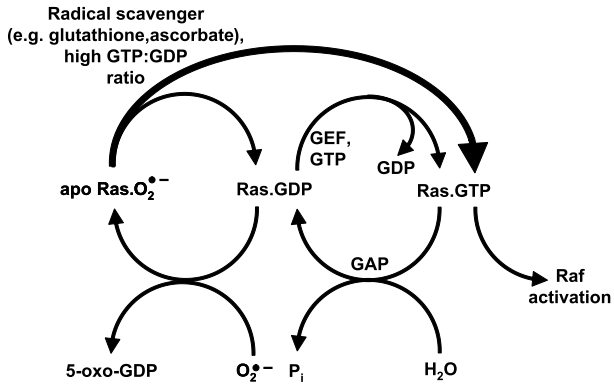

FIG. 4. Activation of Ras. Ras.GDP is biologically inactive. Physiological activation involves exchange of GDP for GTP, a process which is enhanced by guanine nucleotide exchange factors (GEFs). The conformation of Ras.GTP is altered, increasing its affinity for Raf which translocates to the plasma membrane where Ras is localized and the further modifications occurring in that compartment activate Raf, thus initiating activation of the ERK1/2 cascade. The innate GTPase activity of Ras returns Ras to its biologically inactive GDP-ligated state and this hydrolytic activity is enhanced by GTPase-activating proteins (GAPs). In the oxidative activation scheme of Heo and Campbell (66), ROS such as superoxide anions oxidize the bound guanine nucleotide (GDP in this example), and the oxidized species no longer binds to Ras that is converted into a guanine nucleotide-free form incapable of binding guanine nucleotides. In the presence of radical scavengers, Ras again becomes competent to bind guanine nucleotides and hence, depending on the GTP/GDP ratio, Ras may be become activated. In the example shown, the *thicker arrow* is meant to emphasize that, at the normal GTP/GDP concentration ratio in the cell [equal to and in equilibrium with the ATP/ADP concentration ratio which, for total (bound + unbound) ATP/ADP is about 10] and, given the slightly higher affinity of Ras (H-Ras) for GTP over GDP (53), Ras.GTP formation should be favored.

The activation state of Ras was formerly measured directly by metabolic labelling of the guanine nucleotide pools with ^{32}Pi, immunoprecipitation of Ras.GD^{32}P and Ras.GT^{32}P, separation of the guanine nucleotides by thin layer chromatography, and measurement of the GT^{32}P/GD^{32}P ratio. Nowadays, Ras.GTP loading is inferred from the ability of Ras to bind to the recombinant Ras interaction domain of c-Raf coupled to glutathione-*S*-transferase, combined with affinity purification and Western blot analysis (49, 139). However, it should be emphasized that the two methods are not strictly equivalent because it remains theoretically possible for mechanisms to exist which would increase the interaction of Ras with the Ras interaction domain of c-Raf in the absence of Ras.GTP formation. Such interactions might still lead to activation of the ERK1/2 cascade.

In addition to the accepted mechanism involving GDP/ GTP exchange, Ras can be activated in a complex manner by ROS and by reactive nitrogen species (see Fig. 4 for a detailed description). *In vivo*, increasing cardiac oxidative stress by expression of dominant–negative (inhibitory) thioredoxin in transgenic mice increases the ability of Ras to bind to the c-Raf interaction domain (153). Parenthetically, this study (153) also showed an increase in Raf phosphorylation, though it is not clear which phosphorylation site was studied. It should be noted that c-Raf contains both activating and inhibitory phosphorylation sites. For example, phosphorylation of Ser^{259} is probably inhibitory, whereas phosphorylations on Ser^{338} and Tyr^{341} are probably activating (146). The regulation of Ras by ROS has been studied primarily using purified proteins *in vitro* (65, 66, 68, 69, 89). For example, for H-Ras.GDP (Fig. 4), superoxide anions promote GDP dissociation (as 5-oxo-GDP) initially by modifying the Cys^{118} sulphydryl group [which lies in the vicinity of the bound guanine purine ring (50, 62)] to form a thiyl radical and this ultimately generates a form of H-Ras that cannot bind guanine nucleotides (66). In the presence of a suitable radical scavenger (ascorbate or glutathione), guanine nucleotide binding can reoccur. Given the high GTP/ GDP ratio in the cell and assuming that the affinity of Ras for GTP is similar or slightly greater than that for GDP (53), Ras.GTP formation (*i.e.*, activation) would be favored. An analogous scheme applies to activation of Ras by the NO radical (65, 69). In other words, ROS simply cause dissociation of guanine nucleotides from Ras. It is worth noting that such a scheme of activation applies to other small guanine nucleotide-binding proteins such as Rho (67), which has also been implicated in hypertrophic growth of the cardiac myocyte (6, 72).

Sulphydryl groups in proteins are potentially susceptible to glutathionylation (or other modifications) under oxidizing conditions (103), and glutathionylation is reversed by thioredoxins (56, 127). It has recently been shown that α-adrenergic stimulation of adult rat ventricular myocytes decreases the abundance of free Cys-sulphydryl groups in Ras, as assayed by alkylation of lysed cell extracts with biotin-conjugated iodoacetamide (83). Reduced sulphydryl reactivity is also induced by 'specific' [sic, (71)] inhibition of the thioredoxin/ thioredoxin reductase system with azelaic acid [HOOC.$(CH_2)_7$.COOH] which actually inhibits many enzymes in addition to thioredoxin reductase (108), but this is overcome by adenoviral infection of thioredoxin. The authors were able to reverse the α-adrenergic-stimulated reduction in Ras sulphydryl reactivity by including dithiothreitol in their alkylation buffer. This result is surprising because it would be expected that the alkylating agent (biotin-conjugated iodoacetamide) would alkylate dithiotheitol (present in molar excess), thus destroying the alkylating reagent.

With respect to sulphydryl group abundance in Ras, work is more advanced in noncardiomyocytic cells. For example, in COS-7 cells, oxidative stress induced by transfection of a dominant–negative (inhibitory) thioredoxin construct leads to increased *S*-cysteinylation of Ras (153). In rat vascular smooth muscle cells, ROS produced by ANGII-mediated activation of NOX appears to lead to a dithiothreitol-sensitive increase in the c-Raf binding activity of Ras and to glutathionylation of cysteinyl-residues in H-Ras, as identified by mass spectroscopy (2). The same group has reported essentially similar findings with reactive nitrogen species (*e.g.*, peroxynitrite) as oxidant in aortic endothelial cells (36). Adachi *et al*. (2) propose that glutathionylation is associated with an increase in the ability of Ras to bind to the Ras interaction domain of c-Raf, a property usually associated with activated Ras (*i.e.*, Ras.GTP).

There are some unexpected findings reported by Adachi *et al*. (2). The glutathionylated Cys-residues reported are conserved in K-Ras or N-Ras, though differences in the neighboring sequences would allow their unambiguous detection on mass spectrometry. However, no glutathionylated sequences originating from K-Ras or N-Ras were detected (2). With respect to the (glutathionylated) oligopeptide sequences themselves, some still contain the H-Ras C-terminal tripeptide (Val^{187}-Leu^{188}-Ser^{189}). As described above and in Fig. 4, this tripeptide sequence is of necessity cleaved during post-translational processing to produce mature, biologically active H-Ras. Furthermore, the Cys^{186} residue in H-Ras is irreversibly farnesylated as the first step in post-translational processing (Fig. 3) and this would alter the ion mass of the oligopeptide. In other words, it is difficult to understand how the C-terminal, sometimes glutathionylated peptides could originate from mature, biologically active H-Ras.

Furthermore, it is not clear whether the reported glutathionylation of Ras (2) increases Ras.GTP loading. Indeed, because the most crucial site of glutathionylation is Cys^{118} (2, 36, 103) which lies close to the GTP/GDP purine ring binding site (50, 62), glutathionylation is likely to enhance GTP/GDP exchange (A. Wittinghofer, personal communication). Given the high GTP/GDP ratio in the cell, this would lead to the formation of Ras.GTP (*i.e.,* glutathionylation would act as if it were a guanine nucleotide exchange factor). These mechanisms of Ras activation by oxidative stress are clearly somewhat different from that advocated by Heo and Campbell (66). However, a somewhat unexpected finding is that an H-Ras mutant in which Cys^{118} is mutated to Ser (which cannot be glutathionylated) apparently inhibits some of the biological effects of Ras activation (phosphorylation of PKB and p-38-MAPK) in cells exposed to ANGII (2). This inhibition simply would not be predicted unless the mutant species acts in a dominant–negative manner interfering with the biological activity of the (glutathionylated?) endogenous Ras. There is no basis for this suggestion and the H-Ras(Ser^{118}) mutant appears to be indistinguishable from wild-type H-Ras in terms of its biological activity (62). Indeed (and worthy of note), the activation of ERK1/2 (*i.e.*, the best-defined effect of activated Ras) by ANGII is unaffected by H-Ras(Ser^{118}) (2).

CONCLUSIONS

Generally, signaling pathways display a reasonable degree of specificity in terms of their interactions. The conceptual problem with ROS as messenger species is that oxidation reactions are potentially less specific and may be difficult to reverse. High levels of oxidative stress are also clearly detrimental and promote cell death. That being said, there is now such a large body of evidence in favor of ROS acting as a *bona fide* growth-promoting, physiological messenger that the hypothesis cannot be ignored. In resolving this issue of the role of ROS in cell growth and cell death, it is perhaps worth distinguishing between local generation of ROS, possibly downstream from a cell surface receptor, and global generation of ROS as occurs during ischemia with reperfusion. In the former scenario, ROS generation and destruction could be specific and localized (and possibly coupled to receptor activation), and could thus be highly regulated. One problem is that the enzymes best characterized for their activation by receptors (the NOXs) appear to generate extracellular ROS in the form of superoxide anions (88). In the latter situation, ROS production is global and unregulated and is therefore much more likely to be detrimental to the cell. This is probably the situation which can be mimicked by external application of agents such as H_2O_2. Nevertheless, studies of global effects of ROS can provide insights into potential mechanisms by which ROS promotes growth. The two pathways which are particularly implicated in cell growth are the ERK1/2 cascade and PI3K/PKB and, since ROS do activate these pathways, the key question becomes what are the mechanism(s) by which this occurs. Here, we have outlined some of the current theories. In our view, the well-established inactivation of phosphatases almost certainly contributes significantly to the activation (via increased phosphorylation) of signaling pathways, but we allow that other oxidation events may lead to positive activation via proteins such as Ras and PKC.

ABBREVIATIONS

ANGII, angiotensin II; ASK1, apoptosis signal-regulating kinase 1; CSK, C-terminal c-Src kinase; EGF, epidermal growth factor; ERK, extracellular signal-regulating kinase; ET-1, endothelin-1; GPCR, G protein-coupled receptor; IGF1, insulin-like growth factor 1; JNK, c-Jun NH_2 terminal kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MKKK, MKK kinase; NOX, NAD(P)H oxidase; PDK1, 3-phosphoinositide-dependent kinase 1; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PtdIns, phosphatidylinositol; ROS, reactive oxygen species; RPTK, receptor protein tyrosine kinase; SAPK, stress-activated protein kinase or stress-activated MAPK.

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