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COMPREHENSIVE INVITED REVIEW

# Resveratrol: Its Biologic Targets and Functional Activity

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**Abstract**

The polyphenolic phytoalexin resveratrol (RSV) and its analogues have received tremendous attention over the past couple of decades because of a number of reports highlighting their benefits *in vitro* and *in vivo* in a variety of human disease models, including cardio- and neuroprotection, immune regulation, and cancer chemoprevention. These studies have underscored the high degree of diversity in terms of the signaling networks and cellular effector mechanisms that are affected by RSV. The activity of RSV has been linked to cell-surface receptors, membrane signaling pathways, intracellular signal-transduction machinery, nuclear receptors, gene transcription, and metabolic pathways. The promise shown by RSV has prompted heightened interest in studies aimed at translating these observations to clinical settings. In this review, we present a comprehensive account of the basic chemistry of RSV, its bioavailability, and its multiple intracellular target proteins and signaling pathways. *Antioxid. Redox Signal.* 11, 2851–2897.

## I. Sources and Chemistry of RSV

Resveratrol (3,5,4′-trihydroxystilbene, RSV) and its analogues are polyphenolic phytoalexins that occur naturally in many plant species, including grapevines and berries. RSV is composed of two aromatic rings connected by a styrene double bond. In terms of its chemical and physical characteristics, it is a white solid powder with a molecular weight of 228.25 g/mol, low water solubility, a melting point between 253 and 255°C, photo/pH-sensitivity, and the presence of *trans*- and *cis*-isomers, with the *trans*-isomer being the preferred steric form. The p$K_a$ values for the *trans*-isomeric mono-, di-, and triprotonated forms are 9.3, 10, and 10.6, respectively. Both *trans*- and *cis*-RSV can be detected with UV-HPLC at 308 and 288 nm, respectively (271), or with shifts in their NMR spectra (101, 408). The composition of RSV [the number and position of its hydroxyl (OH) groups, its intramolecular hydrogen bonds, its stereo-isometry, and the presence of double bonds] accounts for its activity in biologic systems (308, 385), and Borges *et al.* documented that it is the 4-hydroxystilbene element of RSV that produces an antioxidant effect (331).

Native RSV is thought to play a role in the epidemiologic phenomenon known as the "French Paradox" (339). This term was coined to describe the inverse relation between coronary heart disease mortality and the predominantly red wine consumption seen in France. The validity of this paradox is questionable, as some suggest that the incidence of heart disease in France may have been underestimated and that differences in lifestyle and regions should be considered. A recent study illustrated the large pharmacokinetic variability between subjects and the influence of food on the rate of absorption of RSV, indicating that these factors should be accounted for (13).

However, the suggested benefits of consuming red wine together with a Mediterranean diet have led to a number of studies aimed at understanding the potential benefits of RSV and red wine. With regard to viticulture, Bertelli *et al.* (43) suggested considering the type of wine, the grapes used, the farming region, and the counterbalance effect of the other components in the wine. The World Health Organization (WHO) also highlighted a need to "investigate the possible protective effects of ingredients other than alcohol in alcoholic beverages" (42, 44). Indeed, wines contain many protective and toxic bioactive molecules, such as piceid, polydatin, pinotin A, malvidin 3-glucoside, caffeic acid, and caftaric acid. These points were also stressed in a study on Italian red wines collected and analyzed for both ochratoxin A (cytotoxic) and RSV-related stilbenes (315). The mean level of RSV was characterized as 3.14 mg/L; the level of piceids was 5.80 mg/L; and the level of ochratoxin A, 0.64 mg/L. The Merlot wines showed the highest mean value of total stilbenes, followed by Negroamaro and the Negroamaro blend; Aglianico, and Syrah, all with mean levels of $<$10 mg/L. The highest values of ochratoxin A were in Negroamaro- and Primitivo-based wine samples from southern Italy, which also had the highest content of stilbenes. Thus, the specific type of wine and its RSV level should be carefully considered when studying wine's effect on disease or as a preventive agent. In line with this are the substantial data showing that, depending on the RSV concentration, the RSV has several attributes, including the inhibition of lipid peroxidation and platelet aggregation, metal chelating (primarily copper), free radical–scavenging activity, antiinflammatory activity, modulation of lipid metabolism, and anticancer and estrogenic-like activity (317). These actions allow RSV and its metabolites to thwart cardiovascular, neurologic, and immunologic pathologies and are outlined in this review.

The chemical synthesis of RSV is predominantly carried out by using the Wittig or Wittig-Horner reaction (213), whereas in nature, RSV is synthesized by the enzyme, trihydroxystilbene (RSV synthase) synthase (E.C. 2.3.1.95) (Fig. 1) (24). Intriguingly, RSV and chalcone synthases share a 65–70% sequence homology and the same substrates (124). A molecular switch dictates whether a stilbene or a chalcone is produced, and the site-directed mutagenesis of specific residues can switch the function of a chalcone synthase to that of a RSV synthase (24). The amount of RSV produced is linked to the degree of stress a plant experiences. Should RSV not be required, the plant metabolizes it to the nontoxic piciede. The characterization of RSV synthase has allowed its application in transgenic crop development for the purpose of circumventing the use of artificial pesticides. Interestingly, a growing number of reports detail the enhanced disease resistance conferred by RSV in transgenic crops (see reviews 105, 201). This is based on the antifungal properties of RSV, as RSV is converted to potent antifungal agents such as pterostilbene and viniferin in plants. A recent publication extrapolated this to human health by using bacteria with CYP102A1 mutants to produce the human metabolite of RSV, piceatannol, which has anticancer and antiviral properties (218).

### A. The metabolism and bioavailability of RSV

Metabolomics emerged as a discipline in the 1970s primarily because of the work of Arthur Robinson and Linus

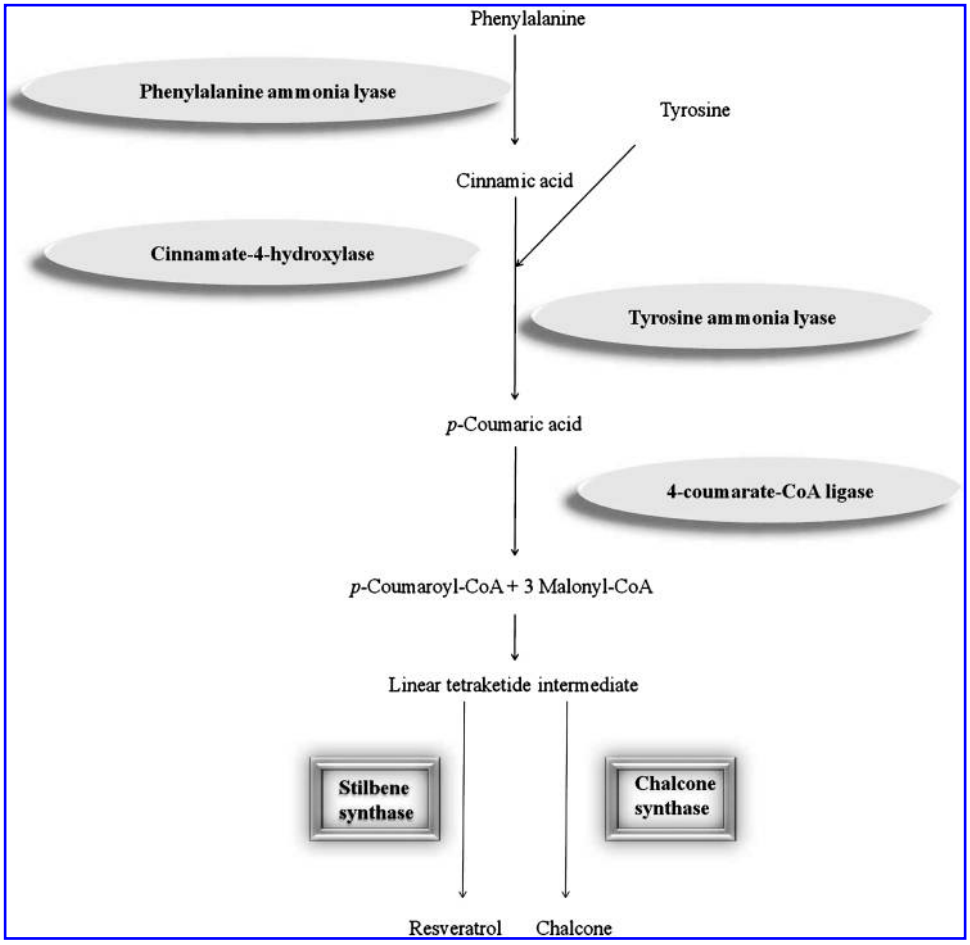



**FIG. 1. An outline of the enzymes involved in the phenylpropanoid pathway that produces RSV or a chalcone, both of which have effective antibacterial, antifungal, antitumor, and antiinflammatory properties.** The initial step involves the conversion of the amino acid, phenylalanine, into *p*-coumaric acid, which then reacts with a pantetheine group of the coenzyme-A to produce 4-coumaroyl-CoA. 4-Coumaroyl-CoA is used by stilbene synthase to catalyze recurring decarboxylative condensation of a *p*-coumaroyl residue to RSV.

Pauling on quantitative and comprehensive analysis of urine and blood metabolites. Xenobiotics are predominantly lipophilic and are converted into more hydrophilic, secretable compounds through biotransformation. This process involves phase I enzymes [*e.g.*, cytochrome P450 monooxygenase (CYP)], which catalyze substrate oxidation, whereas phase II enzymes conjugate polar groups through transferases that attach hydrophilic groups or change the redox state of the molecule (*e.g.*, glutathione-*S*-transferase, UDP-glucuronosyltransferase, sulfotransferase, and NAD(P)H:quinone oxidoreductase) to yield a more water-soluble, secretable product. However, in some instances, procarcinogens are converted into carcinogens because of the formation of reactive oxygen species (ROS) and other damaging molecules, such as semiquinones. These processes result in the depletion of the cellular antioxidant systems, such as those involving glutathione and NADPH/NADP.

Being a lipophilic, phenolic compound, RSV crosses the plasma membrane and is well absorbed when given orally (18). In the body, it is metabolized, and it can interact with and modulate phase I and II enzymes (452). The pharmacokinetic data from laboratory animals and humans points to RSV being rapidly metabolized (especially *via* phase II glucocuronide or sulfate conjugations), which gives it a short half-life of ~8–14 min in the body and, in turn, a low bioavailability (37). Other *in vivo* studies using orally administered RSV show that both the native and conjugated forms appear in the plasma and that RSV can be rapidly absorbed in the colon. Indeed, Boocock *et al.* (270) and Walle *et al.* (423) showed that RSV metabolites could be detected in the plasma for 12–24 h and 72 h, respectively. Additionally, the plasma concentration diminishes until a second spike occurs, suggesting the existence of an RSV "sink," perhaps from bile (270, 423). Another RSV reservoir could also stem from the ability of RSV to bind proteins such as serum albumin or hemoglobin (Hb) at a 1:1 ratio, thereby contributing to RSV being kept in the body for a longer period (262). In humans, the period of excretion appears to be dependent on the plasma concentration of RSV (aglycone and conjugates). However, no direct correlation is found between the amount of RSV administered and the excreted amount (57). Interestingly, the bioavailability of *trans*-RSV is reported to be higher during the morning because of circadian cycles, a fact that will be of interest when considering dosing schedules (13). The recent PREDIMED (Prevencion con Dieta Mediterranea) study also noted that monitoring RSV metabolites in the morning urine is a reliable biomarker of RSV intake (447). Metabolites remain in the plasma much longer than unconverted RSV, whereas methylated RSV remains in the bloodstream for an even longer period, a property that has been exploited in the drug development of RSV analogues. It should also be noted that the major RSV metabolites are polar and thus require specific transportation across the cell membrane. Recent data suggest that this transport can occur

*via* the multidrug-resistance protein 3 (MRP3, ABCC3) or the breast cancer–resistance protein (BCRP, ABCG2) or both, an important observation for the use of RSV in the clinical setting (418).

In mammals, RSV is absorbed in a number of tissues (the liver, the kidney, and the heart) and is found in the plasma, depending on the exposure time and concentration. Studies using Caco-2 (human epithelial colorectal adenocarcinoma) cells have shown that RSV absorption occurs within minutes; the initial absorption of RSV is dose dependent until it plateaus, and the rate-limiting step is the apical-to-basolateral transport (211). Human hepatocytes react in a similar manner, with the initial uptake rate increasing for a few minutes and then remaining stable over a several-hour period (235). Furthermore, RSV (10 $\mu M$) can be rapidly conjugated into monosulfate and disulfate forms and can be entirely metabolized within 8 h in HepG2 (human hepatocellular liver carcinoma) cells (235). These findings are supported by *in vivo* evidence of RSV being transported to the basolateral side, predominantly as a glucuronide and partially as sulfate conjugates (423).

The main metabolites of RSV in mammals are RSV-3-sulfate, RSV-3-*O*-glucuronide, and dihydroresveratrol; however, they are not fully characterized. Recently, Burkon *et al.* (67) used mass spectrometer, HPLC, and NMR analysis of plasma and urine and discovered that, in addition to those already characterized, two new metabolites are detectable and were characterized as novel *trans*-RSV-c/o-conjugated digluocuronides. Thus, as RSV is a pleiotrophic polyphenol *in vivo*, it is likely that some of the activities that contribute to its native state will be found to be because of its metabolites. Several studies have shown that RSV can decrease the expression of the inducible cytochrome P450 (CYP) enzymes (12). Additionally, RSV blocks the activation of the *CYP1A1* promoter and its gene transcription in human hepatoma cells (86). This inhibition of CYP1A1 is also seen in response to the naturally occurring RSV analogues pinostilbene (3,4′-dihydroxy-5-methoxystilbene), desoxyrhapontigenin (3,5-dihydroxy-4′-methoxystilbene), and pterostilbene (3,5-dimethoxy-4′-hydroxystilbene), which appear to be very potent inhibitors of the catalytic activity of CYP1A1 (277). It has been proposed that analogues bearing substitutions of the hydroxyl groups by methoxy groups exhibit a remarkably stronger inhibitory effect on CYP1A1, as compared with the parent compound. In contrast, the effect of pinostilbene, desoxyrhapontigenin, and pterostilbene on CYP1B1 was comparable to that of RSV. RSV also appears to be a direct inhibitor of CYP enzyme activities and can possibly discriminate between CYP isoenzymes, as it directly inhibits CYP1A1 and CYP1B1, whereas it indirectly inhibits CYP1A2 (75). Jang *et al.* (197) found that RSV reduces the development of preneoplastic lesions in mouse mammary gland cultures and decreases the incidence of tumor formation in mice treated with both the tumor promoter 7,12-dimethylbenz(a)anthracene (DMBA) and the tumor initiator phorbol ester. This is in line with the ability of RSV to prevent the initiation phase of carcinogenesis, as DMBA bioactivation by phase I enzymes is carcinogenic. The enzymes involved are likely to be CYP1A1, CYP1A2, and CYP1B1, which are negatively regulated by RSV *via* the aryl hydrocarbon receptor (AhR) (215, 283, 407). This appears to be due to RSV inhibiting the binding of AhR directly to DNA (49).

However, others have reported that AhR may bind to DNA but that RSV prevents its *trans*-activation at the dioxin-responsive elements of genes, such as *CYP1A1* and interleukin-1beta (*IL-b*) (82). AhR is a cytoplasmic transcription factor bound in the inactive form by chaperones, which dissociate on binding to an aryl compound such as estrogen-based 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). This dissociation allows the dimerization of AhR with AhR nuclear translocator (ARNT) and the nuclear translocation of AhR. In the nucleus, AhR interacts with the xenobiotic response element to induce the expression of CYP1A1, CYP1A2, and CYP1B1, and some phase II enzymes. The transcription of dioxin-AhR target genes requires the activity of $Ca^{2+}$/calmodulin–dependent protein kinase II ($Ca^{2+}$/CaM/CaMKI$\alpha$) (283).

The fact that RSV can modulate CYPs is an important consideration in its use as a therapeutic agent. This is illustrated in several studies, and Kang *et al.* (84) reported that when given orally (but not IV), RSV could increase the availability of nicardipine and decrease clearance in rats. RSV also inhibited P-glycoprotein (P-gp) in MCF-7/ADR cells and CYP3A4 (84). From this line of investigation, the authors suggested that RSV increases nicardipine by decreasing P-gp–mediated efflux or inhibiting intestinal CYP3A4. Along similar lines, Choi *et al*. (174) demonstrated that the absolute bioavailability of diltiazem in rats increases in the presence of RSV (2.5 and 10 mg/kg) because of CYP3A4 and P-gp in the intestine or liver or both being inhibited by RSV. Based on these results, it is warranted to consider the need to adjust drug dosages when RSV is used in the clinical setting or as an *ad hoc* supplement.

The phase II family of detoxification enzymes known as quinone oxidoreductases (NQO1 and NQO2) are also affected by RSV. These enzymes catalyze the two-electron reduction of quinines (*e.g.*, menadione to hydroquinones) (420). This catalysis is devoid of the accumulation of dissociated semiquinones and consequently without the formation of free radicals, thereby reducing damaging oxidative stress. Several studies have examined the relationship of *NQO1* and *NQO2* gene polymorphisms with respect to the risk of developing cancer, and these enzymes are now considered potential chemopreventive and chemotherapeutic targets (343). NQO1 is a predominantly cytosolic detoxifying enzyme. Smaller amounts of NQO1 are found in the mitochondria, endoplasmic reticulum, and nucleus, although its function at these locations is unclear. With knockout mice, NQO2 has been characterized as a melatonin-binding site, suggesting that it has a more-complex biologic function yet to be defined (266). NQO1 is upregulated in response to RSV *via* the activation of NF-E2–related factor 2 (Nrf-2)/antioxidant responsive element (ARE) (177), and the proliferation of melanoma cells can be inhibited by the RSV upregulation of NQO2 (178). Furthermore, RSV has a preference for NQO2 over NQO1, and RSV binds directly to and inhibits NQO2 (68). This indicates that RSV can modulate metabolic free radical production, at least in part, by regulation of these enzymes.

## II. Modulation of Cellular Functions by RSV

The versatility of RSV lies in its diverse targeting of membrane and intracellular receptors, signaling molecules, biogenesis enzymes, oxidative systems, DNA-repair mechanisms, and transcription factors (Fig. 2), as well as in the wide

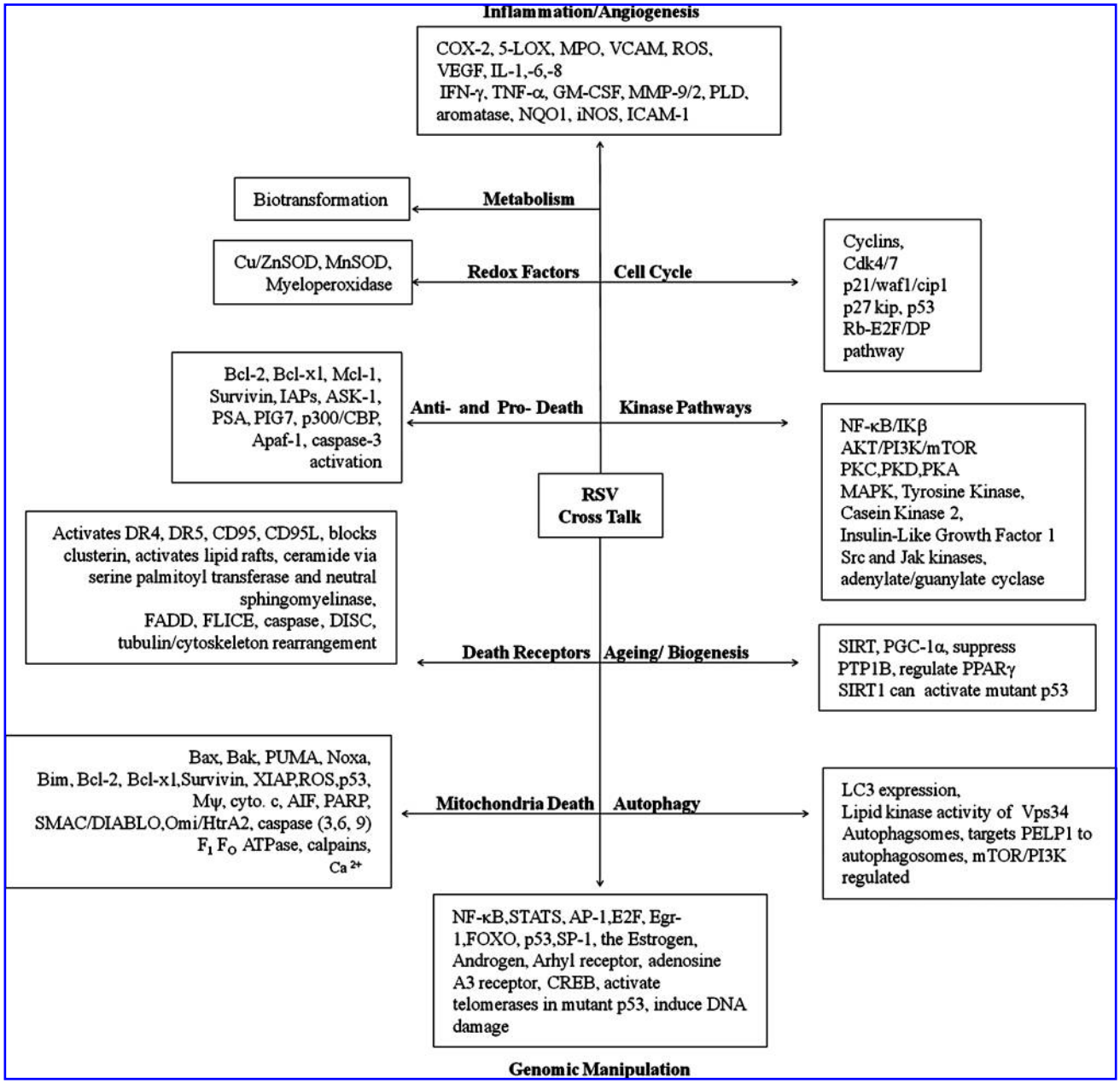


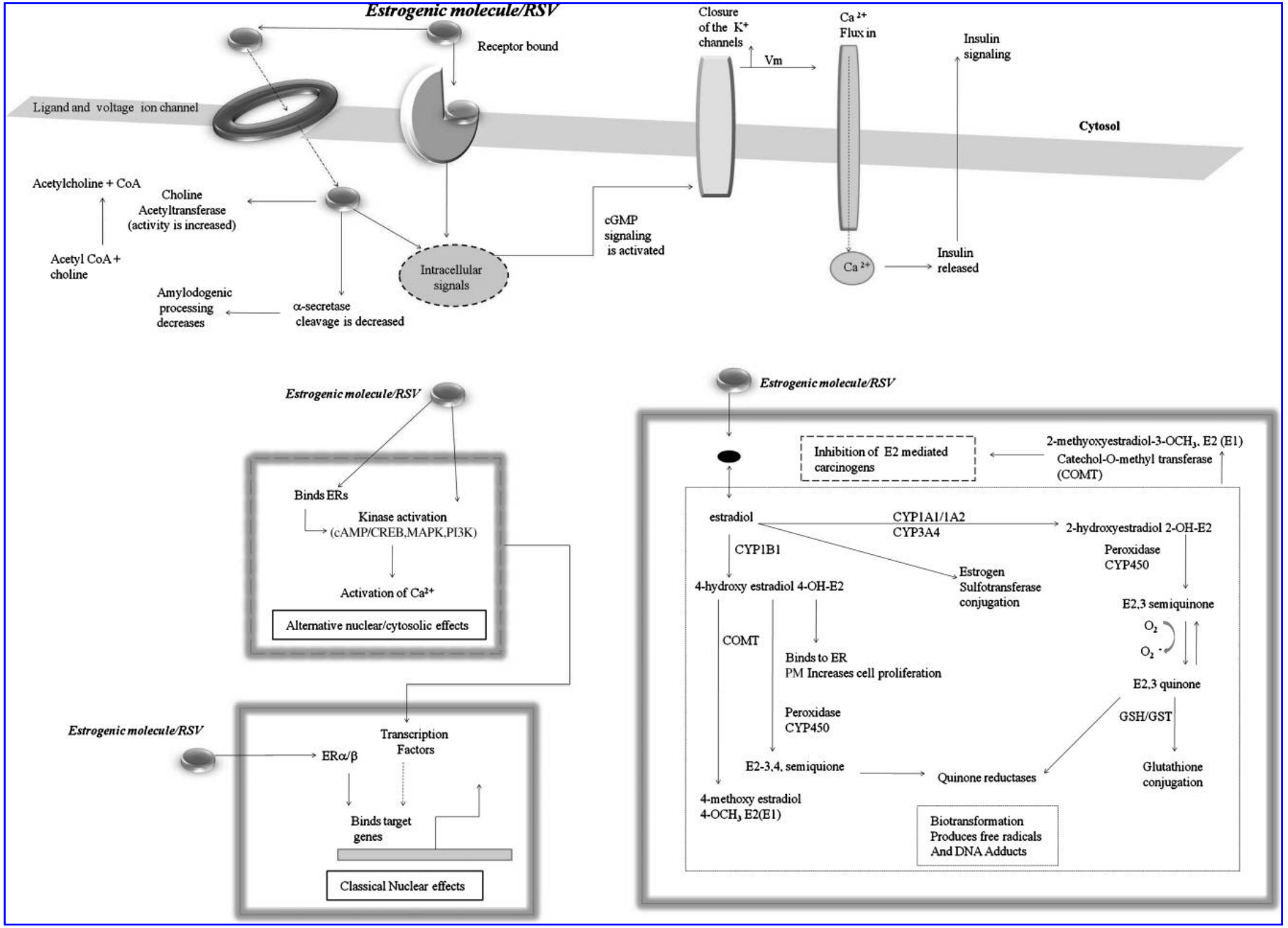

**FIG. 2. A comprehensive list of the various proteins and pathways affected by RSV.** Depending on its concentration, RSV has been reported to exert opposing effects on some biologic systems, such as cell-death signaling, as well as the expression and function of certain proteins (for example, NF-$\kappa$B and the estrogen-receptor pathways).

range of possible RSV-induced effects, including cell proliferation, cell-cycle arrest, differentiation, and cell death. Therefore, while delineating RSV's biological effect(s), it is essential to consider the signaling pathways in the context of the global cellular environment and, more important, the concentration of RSV. In the next few sections, we attempt to discuss some of the important effects of RSV during both normal and altered physiologic states.

### *A. The influence of RSV on signal transduction*

Signal transduction allows a cell to convert a stimulus to a signal. This process is normally an orderly sequence of biochemical reactions that result in a signal-transduction pathway composed of initiators, enhancers, and repressors. RSV has the capacity to activate or repress a number of signal-transducing pathways found throughout the cell. The major ones are discussed later.

1. RSV and hormone signaling. RSV belongs to the type I class of estrogens, which binds estrogen receptors (ERs) with a lower affinity than estradiol (144). Because of its structural similarity and biologic properties, RSV has been compared with 17$\beta$-estradiol (E2), diethylstilbestrol (DES), and certain synthetic xenoestrogens (249). Experimental evidence further supports its superagonist effect in activating hormone


Estrogenic molecule/RSV
Receptor bound
Ligand and voltage ion channel
Closure of the K+ channels
Vm
Ca 2+ Flux in
Insulin signaling
Cytosol
Acetylcholine + CoA
Choline Acetyltransferase (activity is increased)
Acetyl CoA + choline
cGMP signaling is activated
Intracellular signals
Ca 2+
Insulin released
Amylodogenic processing decreases
α-secretase cleavage is decreased
Estrogenic molecule/RSV
Binds ERs
Kinase activation (cAMP/CREB,MAPK,PI3K)
Activation of Ca2+
Alternative nuclear/cytosolic effects
Estrogenic molecule/RSV
ERα/β
Transcription Factors
Binds target genes
Classical Nuclear effects
Estrogenic molecule/RSV
Inhibition of E2 mediated carcinogens
2-methyoxyestradiol-3-OCH3, E2 (E1)
Catechol-O-methyl transferase (COMT)
estradiol
CYP1A1/1A2
CYP3A4
2-hydroxyestradiol 2-OH-E2
CYP1B1
Peroxidase CYP450
Estrogen Sulfotransferase conjugation
4-hydroxy estradiol 4-OH-E2
E2,3 semiquinone
O2
O2 -
COMT
Binds to ER
PM Increases cell proliferation
E2,3 quinone
Peroxidase CYP450
GSH/GST
E2-3,4, semiquione
Quinone reductases
Glutathione conjugation
4-methoxy estradiol 4-OCH3 E2(E1)
Biotransformation
Produces free radicals
And DNA Adducts

receptor–mediated gene transcription (19), thereby giving it a mixed ER agonistic–antagonistic activity in $ER^+$ breast cancer cells (59). These findings provide strong evidence for the classification of RSV as a selective estrogen-receptor modulator (SERM) that can work on the estrogenic signaling pathways (Fig. 3). To this end, exposure to low concentrations of RSV ($<10\,\mu M$) activates phosphoinositide 3-kinase (PI3K) through the estrogen receptor-α (ERα). By using this pathway, RSV ($\geq 50\,\mu M$) elicits a strong inhibitory effect in MCF-7 (breast, adenocarcinoma) cells by downregulating Bcl-2 and nuclear factor-kappa B (NF-κB), increasing ROS and nitric oxide (NO) production, and triggering mitochondria-dependent, caspase-independent death signaling (59, 327, 328).

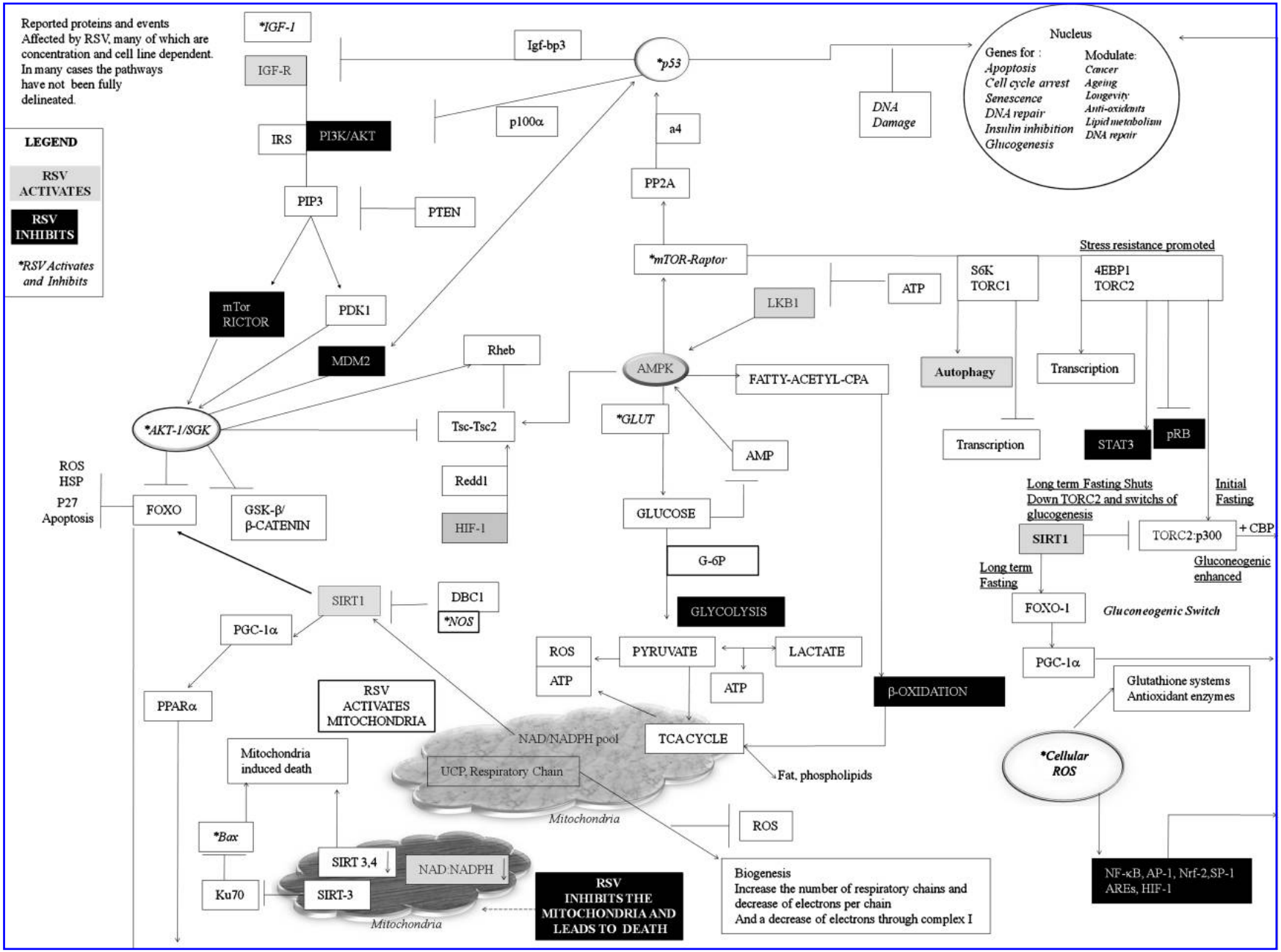
The actions of RSV are not confined to estrogen, as it can also affect the male androgen hormone. RSV can inhibit androgen (R1881)-stimulated growth in LNCaP (androgen-responsive, human prostate carcinoma) cells by decreasing cell viability, clonogenic cell survival, and inducing apoptosis (27). Interestingly, Kuwajerwala *et al.* (232) reported proliferative effects with $5\,\mu M$ of RSV but proapoptotic effects with $>15\,\mu M$ of RSV in androgen-responsive prostate carcinoma cells. The proapoptotic effects in LNCaP cells have been linked to $G_0/G_1$ cell-cycle arrest because of an increased amount of the cell-cycle proteins p53, p21, and p27; changes in cyclins D1 and E, and cyclin-dependent kinase (Cdk 4 and cyclin D1/Cdk4 kinase activity); and an increased apoptotic Bax/Bcl-2 ratio (for further discussion on cell death, see Section III) (39). These changes were not observed in PC3 cells, suggesting that RSV acts differently on steroid receptors.

2. RSV and growth-factor signaling. RSV regulates growth hormone(s) either directly or indirectly. For example, RSV regulates insulin signaling by interacting with insulin and insulin-like growth factor-I (IGF-I), thereby regulating the IGF/growth hormone axis (Fig. 4). RSV can regulate insulin signaling by modifying its secretion as well as its target cells' sensitivity, which could have implications in diabetes and cancer. RSV ($10\,\mu M$) inhibits the expression of autocrine growth stimulators, such as transforming growth factor-alpha (TGF-α) and prostate cancer cell–derived growth factor. RSV can also inhibit IGF-I–receptor mRNA, which may be linked to the estrogen-like properties of RSV (261, 362). The growth factor's link to cancer is exemplified in a study by Harper *et al.* (162) with transgenic adenocarcinoma mouse prostate male (TRAMP) mice. In this study, RSV (625 mg/kg; plasma levels, $53 \pm 18\,nM$) inhibited the development of prostate cancer by modulating IGF-1 signaling. In addition, RSV significantly elevated the expression of the growth inhibitor TGF-β2 mRNA without affecting levels of TGF-β1 or TGF-β3 (392). IGF-II is another growth-related factor that is primarily expressed in embryonic and neonatal tissues and is thought to be a fetal growth factor (129). IGF-II induces autocrine, paracrine, and endocrine activities on bone growth and mass and regulates the sonic hedgehog signaling and angiogenesis (77). The IGF-II receptor (identical to the mannose-6-phosphate receptor) is responsible for the integration of lysosomal enzymes into the lysosomes (142) and for the regulation of cathepsin D, a lysosomal enzyme known to promote breast cancer by destroying regulatory breast tissue (341). Low concentrations of RSV ($1\,\mu M$) can influence this process by increasing cathepsin D and IGF-II secretion in $ER^+$ cells (MCF-7 and T47D) but not in $ER^-$ cells (Hs578t) or normal mammary cells (MCF-10A). High concentrations of RSV ($100\,\mu M$), however, inhibit cathepsin D in $ER^+$ but not in $ER^-$ cells (424). These data support the concentration-dependent, opposing effects of RSV on growth hormone–mediated cancers.

The mitogen epidermal growth factor (EGF) binds to surface-specific, high-affinity, low-capacity EGF receptors (EGFR) (372). Intrinsic to this receptor is its tyrosine kinase activity, which activates signaling cascades such as mitogen-activated protein kinases (MAPKs) that induce the expression and activity of protooncogenes like *c-fos, c-Jun*, and *c-myc*. RSV has been shown to regulate MAPKs through its downstream signaling components (48). The contribution of EGF to tumorigenesis and metastasis is well established, and RSV appears to deregulate EGF signaling (4). This deregulation was demonstrated in MDA-MB-231 (highly metastatic breast cancer) cells in which RSV decreased the chemotactic response by impairing focal adhesions and altering the cytoskeleton (25). This appears to be a dose-dependent mechanism, as low concentrations of RSV ($5\,\mu M$) increased lamellipodia, cell migration, and invasion through a Rac/Cdc42-dependent mechanism, whereas higher concentrations of RSV ($\geq 50\,\mu M$) decreased Rac/Cdc42 activity (26). The RSV-induced modulation of EGFR signaling also affects androgen-independent prostate carcinoma cells (384). Furthermore, in lung cell lines [A549, EBC-1, and Lu65, which have varying degrees of EGFR responses (304)], RSV not only induces an antiproliferative response and apoptosis, but enhances the effect of paclitaxel. In EBC-1 cells, RSV also induces expression of the cell cycle–related protein, $p21^{waf1/cip1}$ (p21) (226).

3. RSV and secondary messengers. Once an extracellular "first messenger" binds to its cognate receptor on the cell surface, a signal "transducer" or a secondary messenger is activated. This triggers chemical and physical reactions and the activation of the intracellular signaling pathways, which converge on the nucleus to activate transcription (184). In this system, the term "secondary messenger" refers to mediators that diffuse from one part of the intracellular space to their spatially removed target. Secondary messengers can be nucleotide based [*e.g.*, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP)], lipid based [*e.g.*, phosphatidylinositol (3,4,5) triphosphate (PIP3) and ceramide], or gaseous [*e.g.*, nitric oxide (NO) and superoxide ($O_2^-$)].

**FIG. 3. A schematic representation of the classic nuclear and nonclassic alternative estrogen-signaling pathways.** RSV can act as an "estrogen mimic," or alternatively, it can displace estradiol by dint of its higher binding affinity, thus activating estrogenic signaling. RSV can also act in an indirect manner by modulating the expression of the biotransforming CYPs involved in the metabolism of estrogen. This decreases the estrogen metabolism as well as the generation of potentially mutagenic semiquinones, which are formed during the biotransformation process. The estrogen-signaling pathway can also modulate the insulin-signaling pathway (Fig. 4), thereby influencing each other.


Reported proteins and events
Affected by RSV, many of which are
concentration and cell line dependent.
In many cases the pathways
have not been fully
delineated.
LEGEND
RSV ACTIVATES
RSV INHIBITS
*RSV Activates and Inhibits
*IGF-1
IGF-R
IRS
PI3K/AKT
PIP3
PTEN
mTor RICTOR
PDK1
MDM2
*AKT-1/SGK
ROS
HSP
P27
Apoptosis
FOXO
GSK-β/ β-CATENIN
SIRT1
DBC1
*NOS
PGC-1α
PPARα
RSV ACTIVATES MITOCHONDRIA
Mitochondria induced death
*Bax
Ku70
SIRT 3,4
SIRT-3
NAD:NADPH
Mitochondria
RSV INHIBITS THE MITOCHONDRIA AND LEADS TO DEATH
Igf-bp3
p100α
*p53
DNA Damage
a4
PP2A
Nucleus
Genes for :
Apoptosis
Cell cycle arrest
Senescence
DNA repair
Insulin inhibition
Glucogenesis
Modulate:
Cancer
Ageing
Longevity
Anti-oxidants
Lipid metabolism
DNA repair
*mTOR-Raptor
Stress resistance promoted
S6K TORC1
4EBP1 TORC2
ATP
LKB1
Rheb
AMPK
FATTY-ACETYL-CPA
Autophagy
Transcription
Tsc-Tsc2
*GLUT
AMP
Transcription
STAT3
pRB
Redd1
HIF-1
GLUCOSE
Long term Fasting Shuts Down TORC2 and switchs of glucogenesis
Initial Fasting
SIRT1
TORC2:p300
+ CBP
Gluconeogenic enhanced
G-6P
Long term Fasting
FOXO-1
Gluconeogenic Switch
GLYCOLYSIS
ROS
ATP
PYRUVATE
LACTATE
ATP
PGC-1α
β-OXIDATION
Glutathione systems
Antioxidant enzymes
NAD/NADPH pool
TCA CYCLE
UCP, Respiratory Chain
Fat, phospholipids
Mitochondria
ROS
*Cellular ROS
Biogenesis
Increase the number of respiratory chains and decrease of electrons per chain
And a decrease of electrons through complex I
NF-κB, AP-1, Nrf-2,SP-1
AREs, HIF-1

The secondary messengers, diacylglycerol (DAG) and inositol triphosphate/inositol 1,4,5-triphosphate (IP3), are generated by the hydrolysis of phospholipids. DAG activates protein kinase C (PKC), whereas IP3 triggers the mobilization of calcium ($Ca^{2+}$) through the activation of the endoplasmic reticulum ligand-gated $Ca^{2+}$ channels. This affects the activities of a variety of target proteins, including protein kinases and phosphatases. The transcription factor cAMP response-element binding (CREB) is phosphorylated by calcium-activated kinase (CaM kinase) and by protein kinase A (PKA). Other examples of intracellular signaling cross-talk include the regulation of adenylyl cyclases and phosphodiesterases by $Ca^{2+}$/calmodulin during the regulation of $Ca^{2+}$ channels by cAMP (247). Therefore, it is thought that the cAMP and $Ca^{2+}$ signaling pathways can cooperate to regulate many cellular responses.

The influence of RSV on $Ca^{2+}$-related pathways is well documented, especially with respect to the cardiovascular system. *In vitro* data suggest that RSV is a potent inhibitor of the $Ca^{2+}$-dependent activities of membrane-associated protein kinase C-alpha (PKC$\alpha$) that are induced by either phorbol ester (12-*O*-tetradecanoyl phorbol-13-acetate, TPA) or DAG ($IC_{50}$, 2 $\mu M$ for both) (375). The inhibition of PKC$\alpha$ activity was reported to be competitive with respect to phorbol ester concentration, but noncompetitive with respect to $Ca^{2+}$ and phosphatidylserine concentrations. This study also showed that RSV inhibited conventional PKC$\beta$, but had no effect on PKC$\varepsilon$ and atypical PKC$\zeta$. The inhibition of total PKC and PKC$\alpha$ appears to be involved in the RSV-induced apoptosis of gastric adenocarcinoma cells (22). However, another study showed conflicting results, as RSV treatment resulted in an increase in the expression of p21 and p53 (and an accompanying S to $G_2$/M arrest) in SNU-1 (human gastric cancer) cells. This effect was preceded by the loss of the membrane-associated PKC$\delta$ protein and a concomitant increase in cytosolic PKC$\alpha$ (23).

Protein kinase D (PKD) is another member of the PKC superfamily and is likewise inhibited by RSV *in vitro* ($IC_{50}$, 200 $\mu M$) as well as *in vivo*, albeit at very high concentrations ($IC_{50}$, 800 $\mu M$) (165). PKD is activated in response to oxidative stress *via* two phosphorylating events (mediated by PKC$\delta$ at Ser738/Ser742 and by Abl kinase at Tyr463) that subsequently trigger a relaying signal to activate NF-$\kappa$B. RSV can inhibit the phosphorylation of PKD at Ser738/Ser742 and thereby repress the translocation of PKD to the IkB kinase complex (IKK), resulting in the inhibition of NF-$\kappa$B activation (384, 386, 413). Consequently, by virtue of its ability to inhibit PKC$\delta$ selectively, RSV can inhibit both PKD and downstream NF-$\kappa$B activation (23, 434).

4. RSV and cAMP/cGMP-mediated signaling. In MCF-7 (a human breast carcinoma) cells, RSV was shown to stimulate adenosine 3′,5′-cyclic monophosphate (cAMP) production ($t_{1/2}$, 6.2 min; $EC_{50}$, 0.8 $\mu M$) *via* a cAMP-dependent kinase (PKA)-mediated mechanism (116). Furthermore, RSV treatment preconditions the heart by activating the adenosine $A_1$ receptor, which activates the latter's effect on the pro-survival PI3K/AKT-Bcl-2 signaling circuit. The PI3K inhibitor LY294002 can partially block the cardioprotective effects of RSV (94). Similarly, RSV activates p38 MAPK, which induces activation of downstream targets, such as AP kinase 2 (AP-2) and mitochondrial lysine-tRNA synthetase (MSK-1). These targets have been implicated in the cardioprotective ability of RSV via preconditioning to ischemia (96). It is worth noting that the adenosine-dependent effects of RSV in protecting from ischemic injury can be blocked by nitric oxide synthase (NOS) inhibitors (60).

Another nucleotide-based secondary messenger is cyclic guanosine monophosphate (cGMP), which is formed from guanosine-5′-triphosphate (GTP) by guanylyl cyclases and is degraded into GMP by a phosphodiesterase. NO and peptide ligands activate guanylyl cyclases, which in turn activate targets such as cGMP-dependent protein kinase/protein kinase G (PKG) (63, 363). Several of the functions of RSV are involved in modulating the cGMP/NO system. For example, RSV increases cGMP levels in coronary arteries, leading to vasorelaxation that remains effective in endothelium-disrupted arteries (115, 128, 346) (see Section IV.A.4). Moreover, acting through the cGMP/NO pathway, RSV can affect the proliferation and osteoblastic differentiation of mouse bone marrow–derived mesenchymal stem cell cultures (382). It should be noted that RSV can also enhance cGMP formation ($t_{1/2}$, 6.3 min; $EC_{50}$, 1.8 $\mu M$) and stimulate PKG activity in human coronary smooth muscle cells *via* a NO/soluble guanylate cyclase–independent mechanism (118). In a subsequent study by using the same cells, it was reported that the PKG system activated by RSV reversed the hydrogen peroxide ($H_2O_2$)-induced activation of extracellular signal–regulated kinase (ERK)1/2, thus preventing coronary arterial proliferation (117). Taken together, these results suggest that using RSV at low dosages may be beneficial in coronary heart disease.

5. RSV and PI3K/AKT (PKB) signaling. Phosphatidylinositol biphosphate (PIP2) is the starting point of a distinct signaling pathway. PIP2 is phosphorylated at the third position of inositol by the enzyme PI3K to yield the second-messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3). A key target of PIP3 is the protein-serine/threonine kinase, AKT/protein kinase B (PKB) that binds PIP3 via the pleckstrin homology (PH) domain of AKT at the plasma membrane. There, AKT is phosphorylated and activated by other PH domain–containing protein kinases. Once activated, AKT phosphorylates a large variety of target proteins, including cell-survival proteins, transcription factors, and metabolic proteins, making it an attractive drug target (171). The other component in this pathway is PTEN (phosphatase and tensin homologue), which has been shown to be mutated in multiple advanced cancers. Historically, the function of PTEN has

**FIG. 4. A simplified depiction of the mutual molecular mechanisms underlying growth-factor signaling with reference to the IGF-1 axis, a major player in the effects of RSV on aging, metabolism, and disease.** This pathway encompasses at least five subgroups of signaling: the PI3K/AKT pathway, the sirtuin pathway, the redox-signaling pathway, the mTOR/AMPK pathway, and p53, and uses a range of posttranslational modifications: phosphorylation, sumoylation, ubiquitination, acetylation, and oxidative modifications such as glutathionation and nitrosylation. The recently described gluconeogenic switch is activated during fasting, and RSV has been shown to switch this off through its activation of SIRT/FOXO.

been described as a phosphatase that negatively regulates PI3K/AKT signaling, although new lines of evidence suggest that it may have other biologic functions (53, 170). There is strong support for the regulatory effect of RSV on PI3K/PTEN–mediated signaling pathway(s). RSV is both an *in vitro* ($IC_{50}$, $\sim 25\,\mu M$) and an *in vivo* ($IC_{50}$, less than $10\,\mu M$) inhibitor of PI3K class IA and its downstream signaling (130). Furthermore, in the LNCaP and PC-3 (prostate hormone–dependent cells) cell lines, RSV ($150\,\mu M$ or less) downregulated the androgen receptor and ERα protein levels and PI3K activity, leading to a decrease in AKT activity and cyclin D expression (40). Similar results were reported using primary tumor cultures. Moreover, in human germinal center–like LY1 and LY18 diffuse large B-cell lymphomas, RSV was shown indirectly to inhibit glycolysis *via* its ability to block the phosphorylation of AKT, p70 S6K, and the S6 ribosomal protein, thus affecting glucose metabolism and ultimately leading to cell-cycle arrest (121). In another study with ovarian cancer cell lines (S2780, CaOV3, SKOV3), exposure to RSV reduced glucose uptake and lactate production, possibly through the modulation of AKT and mTOR, which resulted in an increase in autophagy (223). Similar AKT-inhibitory effects in a breast cancer model treated with RSV were demonstrated, resulting in the activation of caspase-9 (a target of AKT) and caspase 9–dependent apoptosis. These studies underscore the critical regulatory effect of RSV on the PI3K/AKT survival axis.

6. RSV and MAP kinase signaling. The MAPK superfamily is a group of highly conserved signaling molecules, with the best-characterized subfamily being the extracellular signal–regulated kinase1/2 (ERK). The ERK pathway predominantly influences cell proliferation, survival, and differentiation. Opposing this are the other MAPK subfamilies (p38 and JNK), which, depending on the extent and type of stimulus, are primarily related to stress, inflammation, and death (276). All three subfamilies are reported to be agonistically or antagonistically modulated by RSV. This variability is contingent on the cell type and dosage, as illustrated by the findings that RSV, at a concentration between 1 p*M* and $10\,\mu M$, induces phosphorylation of ERK1/2, whereas, at 50–$100\,\mu M$, it inhibits ERK1/2 phosphorylation in neuroblastoma cell lines (279).

Of note, the death-inducing activity of RSV has been linked to its effect on MAPK signaling. For example, RSV treatment induces MAPK/p53-dependent cell death in cervical carcinoma cells (3), and another report demonstrated similar effects in JB6 (mouse epidermal) cells (112, 240, 366, 367). Androgen-independent prostate cancer cells exhibit constitutive activation of ERK1/2, sustained by the EGF/TGFα/EGFR axis as well as other trophic signaling networks (384). It was reported that, in these cells, RSV suppresses the PKC-mediated ERK1/2 activation pathways stimulated by EGF and phorbol esters, resulting in cell growth arrest (384). In the MDA-MB-231 (human breast) cells, RSV induces cell growth and cell-cycle arrest by activating p44/42 MAPK *via* phosphorylation at Thr202/Tyr204 and decreasing the expression of S6 ribosomal protein and its phosphorylation at Ser240/244 (11). In T47D (human breast cancer) cells, RSV decreased the expression of both ribosomal S6 protein kinase p70 S6 kinase (p70S6K) and the phosphorylation of phospho-S6 ribosomal protein (pS6RP), and a time- and concentration-dependent activation of p53 was triggered, accompanied by cell death (10). Interestingly, RSV binds specifically to the αVβ3 integrin receptor in breast cancer cells and triggers the downstream activation of the ERK1/2 pathway, leading to p53 phosphorylation at Ser15, culminating in apoptosis (254). In other model systems, a similar role for ERK1/2 in p53-dependent apoptosis induced by RSV was reported (371). Long-term exposure of tumor cells to low concentrations of RSV has also been shown to induce senescence *via* ROS-mediated pathways involving MAPKs and p53-p21 (169).

7. RSV and Jak/STAT signaling. Originally identified in cytokine receptor signaling studies, the signal transducers and activators of transcription (STAT) proteins are involved in signal transduction (cytosolic function) and the activation of gene transcription (nuclear function). For example, cytoplasmic STAT3 can act as a stathmin antagonizer, thereby stabilizing microtubules (252). STATs can also form "statosomes," heteromeric complexes containing accessory proteins, which, in turn, can "present" specific STATs to the plasma membrane–receptor complex (360). STAT3 is specific in its activation by interferon alpha (IFN-α), but not IFN-β. It works through c-Jun and cAMP response element binding (CREB), and, interestingly, STAT3 is unique among the STAT proteins, as it is the only one that is embryonic-lethal at day 6 to 7 of fetal development in a null phenotype. Recent data from Larner *et al.* (424) demonstrated that STAT3 plays a functional role in mitochondria in regulating the complexes I and II of the electron-transport chain (ETC). STAT3 has been shown to regulate *p53* by binding to its promoter and affecting p53 response genes, and reciprocally, STAT3 inhibition increases p53-mediated apoptosis in tumor cells (299). This explains the advantage conferred by the constitutive activation of STAT3 in tumor cells. Therefore, suppression of STAT3 has emerged as a promising approach for the reactivation of p53. In this respect, it is noteworthy that RSV has been shown to inhibit Src tyrosine kinase activity, thus blocking the Src-STAT3 axis and inducing cell-cycle arrest and apoptosis (224). A known transcriptional target of STAT3 is the prosurvival protein, clusterin, which, when overexpressed, can inhibit activated Bax and confer resistance to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) in tumor cells (448). Clusterin was shown to be a target of RSV in a study demonstrating that the TRAIL-sensitizing action of RSV is due to RSV's transcriptional downregulation of *clusterin* in tumor cells (348). Furthermore, RSV has also been shown to abrogate the phosphorylation of STAT3 in interleukin (IL)-6–treated endothelial cells in a dose- and time-dependent manner, which is linked to its ability to induce NO production through endothelial nitric oxide synthase (eNOS) (439). This study also demonstrated that Rac was involved in the STAT3/IL-6–induced intercellular adhesion molecule (*ICAM-1/CD54*) gene expression, which could be countered via the regulation of STAT-3 by RSV.

8. RSV and ion channels. In the case of ion channels, the main channels influenced by RSV are the large-conductance calcium- and voltage-activated potassium channels ($BK_{Ca}$), the Kv and ATP-sensitive $K^+$ ($K_{ATP}$) channels, voltage-gated calcium channels (VGCCs), and sodium channels. Because of the overlap between organelle membranes, such as illustrated by the mitochondria-associated membranes (MAM) between

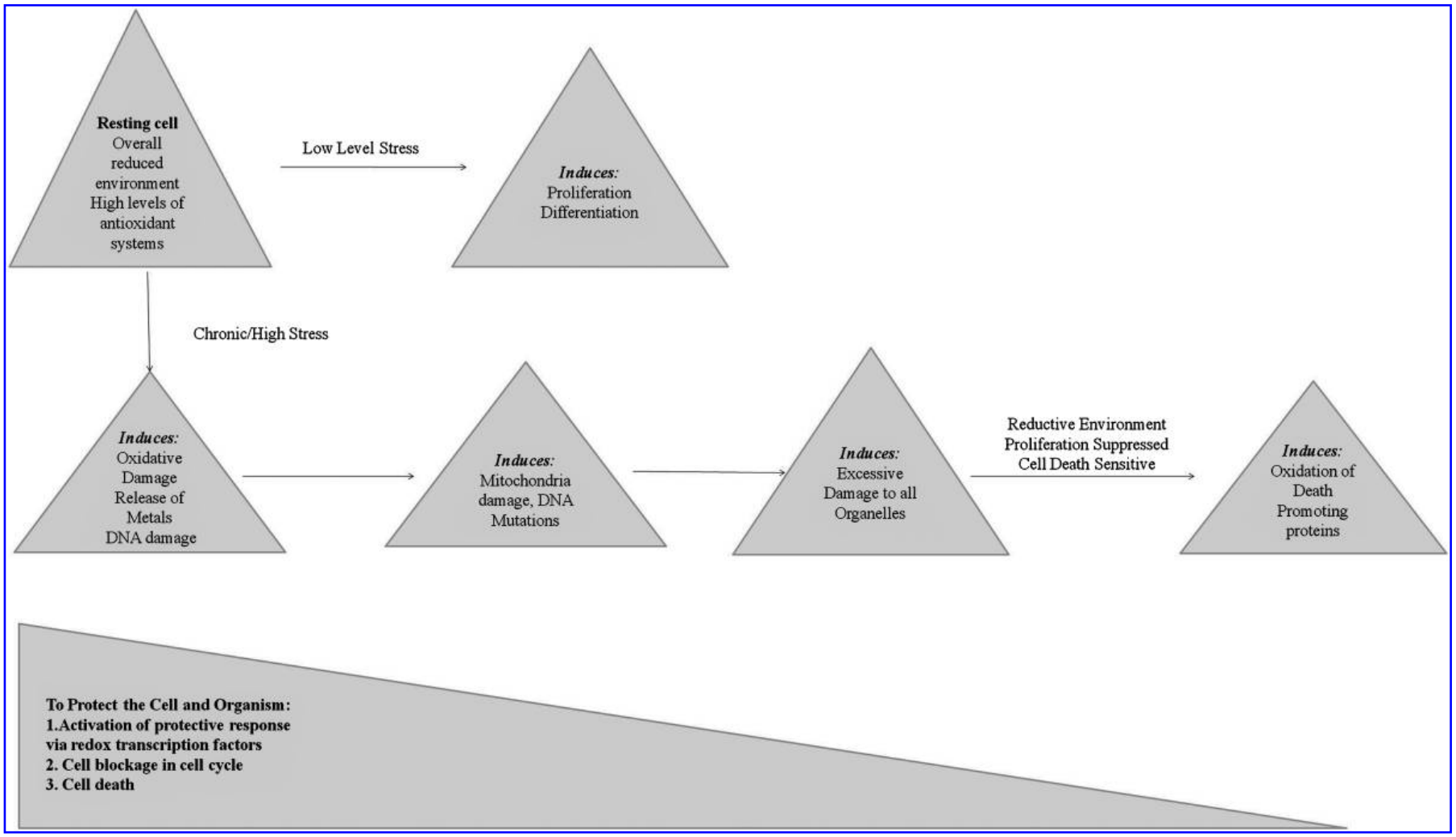
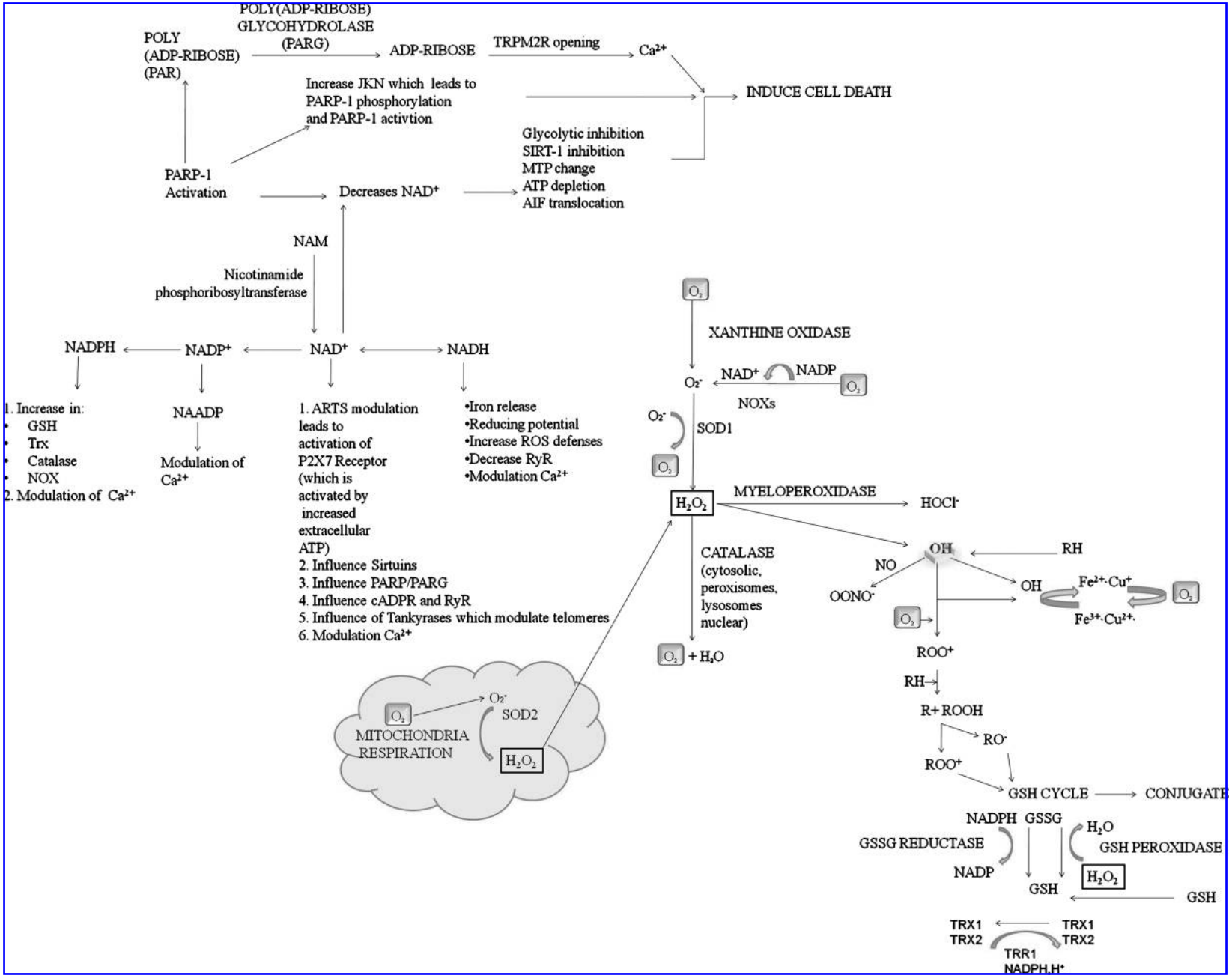



**FIG. 5. An outline of the basis for the "Oxidant Theory of Cancer."** The balance of ROS species and the types of ROS species heavily influence the activity of proteins and the rates of mutations found in cancers.


POLY(ADP-RIBOSE) GLYCOHYDROLASE (PARG)
POLY (ADP-RIBOSE) (PAR)
ADP-RIBOSE
TRPM2R opening
Ca2+
INDUCE CELL DEATH
Increase JKN which leads to PARP-1 phosphorylation and PARP-1 activtion
Glycolytic inhibition
SIRT-1 inhibition
MTP change
ATP depletion
AIF translocation
PARP-1 Activation
Decreases NAD+
NAM
Nicotinamide phosphoribosyltransferase
NADPH
NADP+
NAD+
NADH
1. Increase in:
• GSH
• Trx
• Catalase
• NOX
2. Modulation of Ca2+
NAADP
Modulation of Ca2+
1. ARTS modulation leads to activation of P2X7 Receptor (which is activated by increased extracellular ATP)
2. Influence Sirtuins
3. Influence PARP/PARG
4. Influence cADPR and RyR
5. Influence of Tankyrases which modulate telomeres
6. Modulation Ca2+
•Iron release
•Reducing potential
•Increase ROS defenses
•Decrease RyR
•Modulation Ca2+
O2
XANTHINE OXIDASE
O2-
NAD+
NADP
NOXs
SOD1
H2O2
MYELOPEROXIDASE
HOCl-
CATALASE (cytosolic, peroxisomes, lysosomes nuclear)
O2 + H2O
OH
RH
NO
OONO-
Fe2+·Cu+
Fe3+·Cu2+·
ROO+
RH
R+ROOH
RO·
ROO+
GSH CYCLE
CONJUGATE
NADPH
GSSG
H2O
GSSG REDUCTASE
GSH PEROXIDASE
NADP
GSH
GSH
TRX1
TRX2
TRX1
TRX2
TRR1
NADPH,H+
MITOCHONDRIA RESPIRATION
SOD2

the mitochondria and the ER, it is often difficult to obtain a clear picture of their localization. It does, however, appear that mitochondrial mtBK$_{Ca}$, mtK$_{ATP}$, and Kv channels exist, as well as those localized at the plasma membrane.

Kv channels are potassium ($K^+$) as well as voltage regulated and localized to the inner mitochondria membrane. Involvement of these channels in T-cell activation, in cell-cycle entry and progression, and in the process of carcinogenesis, has been demonstrated. In addition, Kv1.3 inhibitors elicit strong immunosuppressant effects. RSV has been shown to induce smooth muscle relaxation in human internal mammary artery, rat aorta, and rat mesenteric artery *via* activation of margatoxin-sensitive voltage-dependent $K^+$ (Kv) (148, 301). In contrast, Orsini *et al.* (307) and Chen *et al.* (80) suggested that RSV derivates inhibited Kv channels in different experimental models. RSV has also been shown to influence the K$_{ATP}$ channel, which is rapidly inhibited by intracellular ATP. The K$_{ATP}$ channel is formed from an inwardly rectifying potassium (K$_{ir}$6.2) pore-forming subunit with and without the sulfonylurea receptor (SUR) regulatory subunit. ADP, diazoxide, and PIP2 can increase the open state of these channels while ATP inhibits it, and the adenylate kinase system plays a major role in regulating the activation state of these channels. RSV has been shown to act as a K$_{ATP}$ blocker.

RSV also affects the large-conductance $Ca^{2+}$ and voltage-activated $K^+$ channels (BK$_{Ca}$). BK$_{Ca}$ channels are important in the regulation of pulmonary arterial pressure, and inhibition of the BK$_{Ca}$ channel has been implicated in the development of pulmonary hypertension. RSV can influence these channels by its ability to release $Ca^{2+}$ from intracellular stores, as well as by directly stimulating BK$_{Ca}$ channel activity in vascular endothelial cells (148). These channels also play an important role in the regulation of cerebral pressure as well as in the release of neurotransmitters. In neurologic models, RSV has been shown to protect against cell injury produced by oxygen depletion or $H_2O_2$ through the activation of BK$_{Ca}$ channel.

### *B. RSV as a modulator of cellular redox status*

Free radicals are derived from gases and metals, and their chemical structures display one or more unpaired electrons. In understanding whether free radicals will be a destructive force or a necessity for cellular function, their activity must be understood within the framework of the biochemical environment in which they are found, as suggested by the oxidative theory of cancer (Fig. 5). Therefore, it is appealing to add free radicals to the list of cell-cycle modulators (164). Detoxification systems for these free radicals can be enzymatic (scavengers of free radicals: catalase, superoxide dismutase, peroxiredoxins, glutathione peroxidases, glutaredoxins, ferritin, and ceruloplasmin; or reversers of free radical modifications: peroxisomes and thioredoxins) or nonenzymatic [vitamins E and C and the GSH/GSSH (reduced/oxidized glutathione) ratio]. The now-famous term "oxidative stress" was initially coined to refer to an imbalance between these pro- and antioxidant forces, which could be potentially hazardous to biologic systems. Attention has been drawn to the fact that the major cellular thiol/disulfide systems, including the philothione GSH/GSSG, thioredoxin-1 (-SH(2)/-SS), and Cys/CySS, are not in redox equilibrium and respond differently to chemical and physiological stimuli. Thus, the "balance" of pro- and antioxidants cannot be defined by a single entity (205). Accordingly, at least from a mechanistic standpoint, oxidative stress may be better defined as a disruption of redox signaling and control. In the context of free radical biology, RSV has been postulated to act as an antioxidant because of its ability to (a) compete with co-enzyme Q and decrease mitochondria ROS production, (b) scavenge superoxide radicals, (c) inhibit lipid peroxidation induced by Fenton reactions, and (d) regulate the expression of antioxidant cofactors and enzymes (Fig. 6).

1. RSV and oxidative stress. Oxygen-centered free radicals are also known as reactive oxygen species (ROS). These species can modify proteins, lipids, carbohydrates, and nucleic acids. Additionally, proteins can be modified by highly reactive aldehydes and ketones that are produced during the ROS-mediated oxidation of lipids. These aldehydes and ketones are produced either as part of a detrimental system or as a component of signaling modification and have been proposed as "oncogases" because of their involvement in cancer development (318).

In animals, 85–90% of oxygen ($O_2$) intake is used by the mitochondrial cytochrome *c* oxidase in the oxidative phosphorylation reaction required for ATP synthesis. Of this intake, 2% generates $O_2^-$, whereas 0.5% generates NO (345). NO and $O_2^-$ are competitors for cytochrome *c* oxidase, with NO acting as a negative regulator to maintain the intramitochondrial steady state of NO levels at 20–25 n*M*. The remainder is used in the oxidative biosynthesis of vasoactive substances such as prostaglandins and secondary gaseous mediators [*e.g.*, carbon monoxide (CO)]. The second source of $O_2^-$ is the NADPH oxidase family (NOXs), which transport electrons across membranes to generate $O_2^-$ (191, 445).

The cell rids itself of these potentially detrimental levels of free radicals *via* its antioxidant systems. $O_2^-$ is predominantly converted into the less-reactive $H_2O_2$ by the activity of enzymes such as superoxide dismutase (SOD). However, it is also extremely efficient at reacting with NO and with metal centers [*e.g.*, hemoglobin iron-sulfur (Fe-S) clusters]. It is also important to note that not all $H_2O_2$ is derived from $O_2^-$, a fact overlooked by many researchers in this now "trendy" field. Indeed, numerous bivalent enzymes can produce $H_2O_2$. In living cells, the level of $O_2^-$ is influenced by (a) the partial pressure of oxygen ($po_2$); (b) the redox cycling compounds; (c) the $O_2^-$-producing enzymes (*e.g.*, xanthine oxidase); (d) the activity level of the mitochondria electron-transport chain; and (e) reactions with other species, such as NO and sulfur.

RSV has a particularly intriguing duality in buffering the oxidant system. In free radical electrochemistry, the oxidation of RSV is a complex, irreversible, pH-dependent process, which generates two oxidation peaks corresponding to a phenol and a resorcinol moiety. Both of these are major players in the induction of cell death in a variety of tumor cells (385). Conversely, RSV is a robust scavenger of $O_2^-$, hydroxyl

**FIG. 6. A summary of the redox-based pathways affected by RSV either directly or indirectly.** The balance of the cellular NADPH system influences the cellular sensitivity to redox stress.

($^{\bullet}OH$), peroxynitrite ($ONOO^-$), and metal-induced radicals. A rate constant for the RSV reaction with the $^{\bullet}OH$ radical was calculated to be $9.45\times10^8\,M^{-1}\,s^{-1}$ in RAW 264.7 monocytes (245). It should also be noted that a RSV dimer found in wine, pallidol, is a potent (rate constant, $K_a = 1.71\times10^{10}$) and selective scavenger of singlet oxygen (167). It has been shown that pretreatment of HL-60 (promyelocytic leukemia) cells for 2 h with a low dose of RSV (4–8 $\mu M$) results in a slight prooxidant state (a moderate increase in intracellular $O_2^-$ levels), thereby preventing $H_2O_2$-induced acidification, caspase activation, and apoptosis (8). Conversely, high doses of RSV cause the production of free radicals, leading to a reduced intracellular milieu, which is primed for the induction of cell death (7). RSV has also been shown to induce NADPH-dependent $O_2^-$ production in a manner that is linked to PI3K activation (325). RSV is also described as a regulator of the antioxidant and metabolic enzymes, such as phase II detoxification enzymes, *via* direct enzymatic modification or adjustment of the redox-controlled gene transcription through the transcription factors, activator proteins 1 (AP)-1 and 2, Nrf-2, forkhead box O-class (FOXO), and SP-1. RSV can also affect the transcription of antioxidant enzymes and can cross the blood–brain barrier in rats. Intraperitoneal administration of RSV decreases the brain's malondialdehyde level, while increasing the enzymatic activities of SOD, catalase, and peroxidase (282). RSV has been shown to increase MnSOD (SOD2/mitochondrial SOD) in the brain when administered in several ways, suggesting that it has neuroprotective abilities through modulation of the antioxidant systems (340). Robb *et al*. (340) proposed that this increase in MnSOD is due to RSV activating $NAD^+$/sirtuin, which in turn, allows activation of FOXO3a. MnSOD is a target of FOXO3a, and MnSOD expression is strongly induced in cells overexpressing FOXO3a.

2. RSV and NO. Nitrogen monoxide (NO, nitric oxide) is a short-lived diatomic free radical able to diffuse across membranes. It can act in a paracrine or autocrine fashion, depending on the intracellular redox state, to result in vasodilatation, inhibition of platelet adhesion and aggregation, regulation of adhesion molecule expression, suppression of cell growth and migration, and induction or inhibition of cell death (403). NO is produced by the monooxygenase nitric oxide synthase (NOS) from arginine with the aid of $O_2$ and nicotinamide adenine dinucleotide phosphate (NADPH). As with many signaling molecules, the physiologic effect of NO is concentration dependent, with the constitutive isoforms of NOS (endothelial, neuronal, and mitochondrial NOS) providing low intracellular concentrations of NO. An inducible form of NOS (iNOS) is activated at the transcriptional level during inflammation by the transcription factors NF-$\kappa$B, AP-1, CREB, and STATs generating high titers of NO (316).

In normal cells, micromolar concentrations of RSV induce vascular smooth muscle relaxation by promoting the release of NO *via* indirect pathways in a process that is counteracted by NOS inhibitors. In cardiac fibroblasts, RSV appears to inhibit the actions of angiotensin (ANG)-II *via* a NO-cGMP signaling pathway that can be inhibited by *N*-nitro-L-arginine methyl ester (L-NAME) (429). In vascular endothelial cells, RSV suppresses the effect of oxidized low-density lipoproteins (oxLDLs) by inhibiting the association of $gp91^{phox}$ and Rac1, as well as inhibiting ANG-II–induced NADH/NADPH oxidase (NOX) activation (85). Furthermore, the metabolic inactivation of NO by NOXs is inhibited by RSV in cardiac protection, and the expression of eNOS is associated with the activation of p53 and p21 and $S/G_2$ cell-cycle arrest (306). Similarly, in lipopolysaccharide (LPS)-stimulated macrophages, RSV reduces iNOS mRNA transcription and cytosolic protein levels, possibly by blocking the phosphorylation of IKK$\beta$ and subsequent activation of NF-$\kappa$B (243, 263, 411).

NOS expression is often associated with the induction of tumor markers such as cyclooxygenase-2 (COX-2), vascular endothelial adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1, CD54) (253). RSV modulates the expression of these tumor markers through its effect on iNOS expression and activation (333, 342). This bimodal regulation of the NO/NOS system by RSV has been associated with the S-phase arrest in T47D (hormone-sensitive breast cancer) cells (93, 297). This is also seen in other cell lines (*e.g.*, HepG2) in which RSV is a potent inhibitor of cell proliferation. It can reduce the production of ROS and induce apoptosis through cell-cycle arrest in $G_1$ and $G_2$/M phases *via* the activation of NOS (300).

3. RSV and thiol-based redox systems. Methionine and cysteine are sulfur-containing amino acids and, as such, are very sensitive to oxidation. Oxidative modification of these amino acids can alter the physiologic activity of a protein. *In vivo*, the sulfur in cysteine can be found between a −2 (thiol) and +4 (sulfonic acid) oxidation state, whereas other sulfur-based biomolecules contain sulfur in a higher (+6) oxidation state (195). Selenocysteine-containing proteins [*e.g.*, glutathione peroxidases (GPX), tetraiodothyronine 5′ deiodinases, NADPH thioredoxin reductase (TrxR), formate dehydrogenases, and glycine reductases] posses a selenium atom in place of the sulfur in cysteine. Some chemical conversions are reversible, thereby acting as "sensors," although others are irreversible. Glutathionylation, sulfenyl-amide formation, and disulfide activation are considered relatively simple redox processes. Processes such as the "sulfinic acid switch" undertaken by peroxiredoxin enzymes are more complex and involve sulfenic and sulfinic acids that interact with various thioredoxin systems [thioredoxin (Trx), NADPH thioredoxin reductase (TrxR), sulfiredoxin, and peroxiredoxins] (223). These thiol-dependent/thiol-disulfide exchange reactions are crucial to the control of the reduced intracellular redox environment (17, 206). High Trx levels are linked to chemotherapy resistance (138), and TrxR may be essential for the carcinogenic process and for the invasive phenotype of cancer (17, 210). The reverse is also seen with high levels of Trx and TrxR, suggesting that they induce apoptosis and reduce the mitotic index in certain tumors in a p53-dependent manner (275, 359). Their ability to bind to signaling molecules, such as apoptosis signal-regulating kinase-1 (ASK-1) (380, 381) and thioredoxin-interacting protein (Txnip) (369, 441), is probably also involved in determining the cellular resistance to cell death.

RSV affects the enzyme-expression levels of these systems. This is exemplified in A549 (non–small cell lung cancer) cells, in which RSV can induce an increase in selenophosphate synthetase 2 and SOD2 (MnSOD), whereas mRNA levels of Trx, TrxR, glutathione reductase, glutathione *S*-transferase, SOD1 (Cu/ZnSOD), and catalase are unaltered (179). In this study, RSV was also found to upregulate p53, CD95, and Bcl-2, whereas the mRNA level of survivin was reduced.

Another study showed that RSV can also enhance myocardial angiogenesis *in vivo* and *in vitro* by induction of vascular endothelial growth factor (VEGF) in a process that is regulated by Trx-1 and heme oxygenase-1 (HO-1) (209). In cardiac preconditioning, RSV triggers NO-dependent upregulation of MAPKK (MAPK kinase), heat-shock protein (Hsp) 27, and the phosphatidylethanolamine (PE) binding protein (45). Interestingly, a "Trx-2–dependent switch" dictating cell-fate decisions in ischemic rodent hearts has been described (97). RSV may act as a protective antioxidant through modulation of this "switch." That is, by virtue of its ability to prevent GSH/GSSH depletion and induce the expression of Trx-2 (mitochondrially localized Trx), RSV increases the half-life of NO coupled with other pathways.

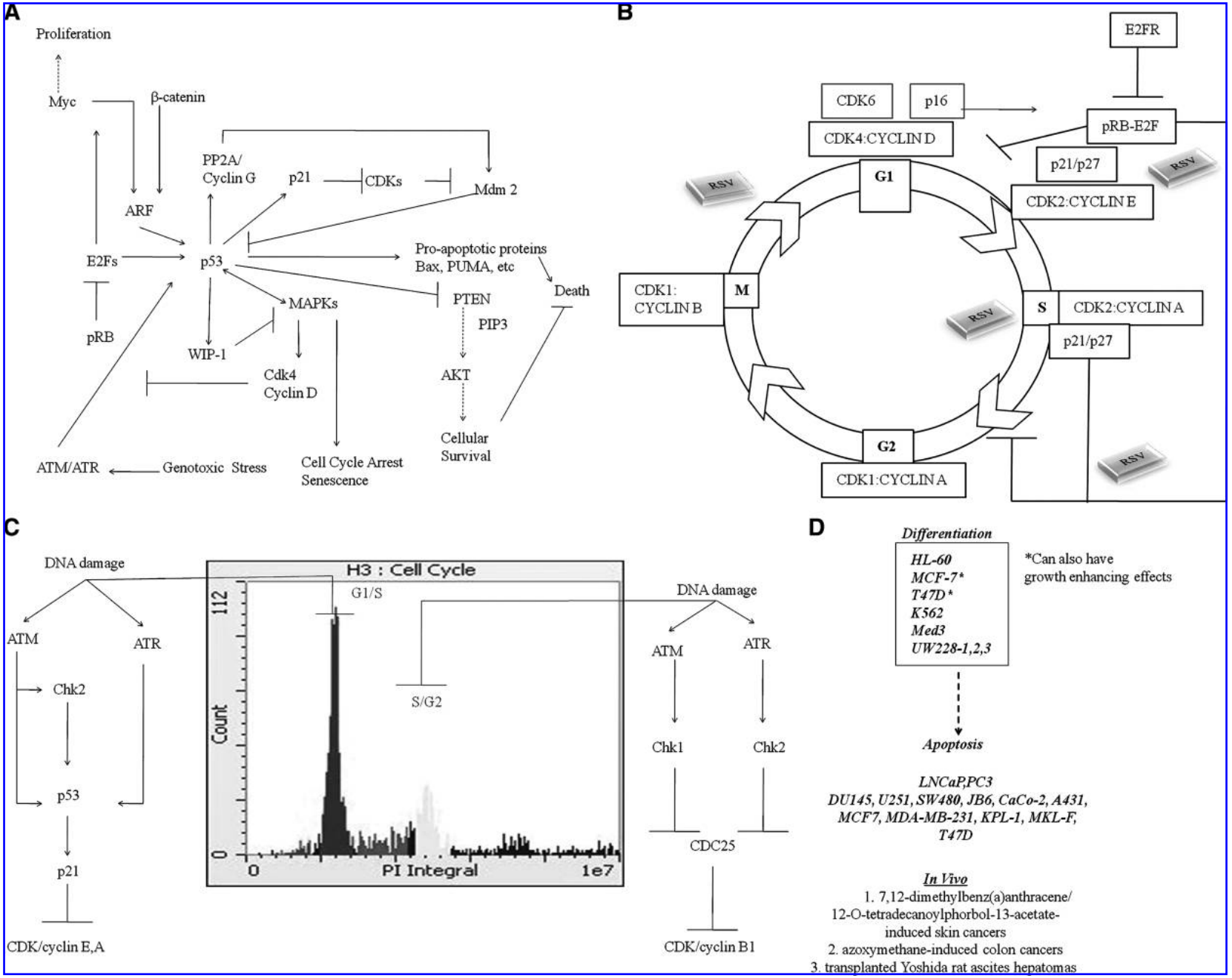
### *C. RSV-mediated control of the cell cycle and gene expression*

As mentioned earlier, micromolar concentrations of RSV induce growth arrest, particularly at the boundary between the S and $G_2$/M phases (Fig. 7). The outcome of the cell-cycle arrest is variable, ranging from cell senescence to differentiation or cell death. However, the transcriptional control of a number of proteins related to the p53 family is a common theme in cell-cycle regulation by RSV.

1. RSV and p53. The protein p53 is a tumor suppressor with a broad mutation spectrum; 20,000 mutations so far have been characterized by the International Agency for Research on Cancer (IARC) (320). As a prominent protein involved in genetic stability and cellular homeostasis, p53 regulates cell growth in response to DNA damage, oncogene activation, and stress (159, 199). Thus, a functional p53 can act as a cellular growth restrictor by inducing senescence, cell-cycle arrest (at $G_1$ or $G_2$ phase or both), or apoptosis. It likely carries out this function through its capacity to bind to target gene promoters at both canonic and noncanonic in sequences. This binding process requires the stabilization and enhancement of its DNA-binding and transcriptional activity through extensive posttranslational modifications (*e.g.*, acetylation, sumoylation, phosphorylation, and protein–protein interactions with cofactors).

The p53 protein is a target of RSV, which was first shown to induce p53-dependent cell death in the JB6 (mouse epidermal) cell line (179). Since then, numerous reports investigating the effects of RSV on p53 and the expression of related cell cycle–specific proteins have been published. Overall, the actions of RSV result in an altered proteome, modulating Cdks and cyclins at the transcriptional and posttranscriptional levels, resulting in specific cell-cycle phase arrest. One such protein induced by stress *via* both p53-dependent and -independent mechanisms is the cyclin-dependent kinase inhibitor p21 (255). This is exemplified in pulmonary artery endothelial cells, where RSV inhibits proliferation by suppressing S- and $G_2$-phase cell-cycle progression through an increase in p53 expression and elevated p21 levels (64). Experimental evidence also suggests that the increase in p53 levels on exposure to RSV correlates with the appearance of posttranslational modifications (phosphorylation and acetylation) required for the direct transcriptional activation of p53-responsive genes related to cell-cycle arrest and apoptosis (*e.g.*, *p21*, *p300/CBP*, *Apaf-1*, and *Bak*), and downregulation of tumor-associated antigens [prostate specific antigen (PSA), NF-$\kappa$B/p65, and Bcl-2] (292–294). In addition, RSV can control the expression of the coactivator p300 (an acetyl-transferase), which is a regulator of p53 (237, 293, 294, 400). It is therefore plausible that p300 and p53 belong to a gene-regulatory loop involved in RSV-induced apoptosis. Conversely, RSV can reversibly activate p53 *via* serine phosphorylation without increasing the expression of p53 or p21 in vascular endothelial cells, and this process causes a nonlethal blockage of DNA synthesis (156). In a similar endothelial cell model, RSV-dependent p53 and p21 activation and cell-cycle arrest have been documented, but only in proliferating serum-stimulated cells and not in quiescent cells (280). In addition, p53 phosphorylation by c-Jun NH(2)-terminal kinases (JNKs) has been reported on exposure to relatively low levels of RSV (10–40 $\mu M$) (367). This is the same concentration responsible for the inhibition of tumor promoter–induced cell transformation and the activation of ERK- and p38 MAPK-mediated apoptosis (366). In DU145 (prostate cancer, hormone-insensitive) cells, RSV has been reported to induce apoptosis *via* MAPK-mediated phosphorylation of p53 on Ser15, with an accompanying increase in p21 expression and decrease in EGF expression (371). In this study, EGF activation of MAPK inhibited the effects of RSV. Conversely, the inhibition of EGF enhances RSV-induced ERK1/2 activity *via* a PKC-dependent mechanism (which does not appear to enhance p53 phosphorylation). In contrast, in LNCaP (hormone-sensitive) cells, EGF suppresses the RSV activation of PKC *via* a p53/MAPK-dependent mechanism, showing that the genetic background of the cell plays an important role in determining the response triggered by RSV.

2. RSV and the retinoblastoma protein. Another major cell-cycle $G_1$/S "gatekeeper" is the retinoblastoma gene (Rb) gene product, which suppresses gene transcription. It is primarily known as a repressor of gene transcription and is functionally inactive in a number of cancers. In the early $G_1$ phase of normal cells, the un- and/or hypophosphorylated active form of Rb associates with the E2F family of transcription factors, which represses transcription of genes. By the end of the $G_1$ phase, Rb becomes inactive *via* hyperphosphorylation by Cdk/cyclin complexes and dissociates from E2F. Together with its binding partner DP-1, E2F mediates the trans-activation of E2F-1 target genes that facilitate the $G_1$/S transition and continuation of S phase. Lees *et al*. (187) recently demonstrated that pRb has a second role as a promoter of cell death in stress-activated induced apoptosis or cell-cycle arrest *via* E1A-induced stress by using drugs such as doxorubicin or DNA-damaging agents, such as UV. RSV can have multiple effects on Rb, as it has a complexity that is only just beginning to be understood. It has been shown that high concentrations of RSV (100 $\mu M$) in rat vascular smooth endothelial cells leads to an increase in hyperphosphorylated Rb, promoting reversible S-phase arrest (156). This S-phase cell-cycle arrest is reversible and is believed to be mediated by Ser15-phosphorylated p53, although the cyclin inhibitors p21 and p27 decrease as well. Conversely, human epidermoid carcinoma cells treated with relatively low concentrations of RSV (10 $\mu M$) for 24–48 h undergo a decrease in the levels of pRb concomitant with a decrease in E2F protein and activity (1). This could be due to the augmentation of p21, thereby inhibiting the related Cdk/cyclin complex, leading to a $G_0/G_1$


A
Proliferation
Myc
β-catenin
PP2A/
Cyclin G
p21
CDKs
Mdm 2
ARF
E2Fs
p53
Pro-apoptotic proteins
Bax, PUMA, etc
PTEN
Death
MAPKs
pRB
PIP3
WIP-1
Cdk4
Cyclin D
AKT
Cellular
Survival
ATM/ATR
Genotoxic Stress
Cell Cycle Arrest
Senescence
B
E2FR
CDK6
p16
CDK4:CYCLIN D
pRB-E2F
p21/p27
RSV
G1
CDK2:CYCLIN E
CDK1:
CYCLIN B
M
S
CDK2:CYCLIN A
p21/p27
RSV
RSV
G2
CDK1:CYCLIN A
RSV
C
DNA damage
ATM
ATR
Chk2
p53
p21
CDK/cyclin E,A
H3 : Cell Cycle
G1/S
S/G2
112
Count
0
0
PI Integral
1e7
DNA damage
ATM
ATR
Chk1
Chk2
CDC25
CDK/cyclin B1
D
Differentiation
HL-60
MCF-7*
T47D*
K562
Med3
UW228-1,2,3
*Can also have
growth enhancing effects
Apoptosis
LNCaP,PC3
DU145, U251, SW480, JB6, CaCo-2, A431,
MCF7, MDA-MB-231, KPL-1, MKL-F,
T47D
In Vivo
1. 7,12-dimethylbenz(a)anthracene/
12-O-tetradecanoylphorbol-13-acetate-
induced skin cancers
2. azoxymethane-induced colon cancers
3. transplanted Yoshida rat ascites hepatomas

arrest. As an added wrinkle, RSV is able to induce mitochondrial cell death in the Y79 retinoblastoma cells (350), whereas in breast cancer cells, this occurs *via* calcium-dependent calpain protease activation (349). It has been shown in human diploid fibroblasts that the RSV activation of sirtuin-1 (SIRT1) leads to the activation of ERK and S6K1, reduces the expression of $p16^{INK4A}$, and promotes phosphorylation of Rb (182). This pathway promotes cell proliferation and decreases cellular senescence. These findings will be of interest to study further in the context of aging, as a lack of ERK and S6K1 signaling occurs in aged cells.

3. RSV and the early-response factors. When induced, immediate-early genes (*e.g.*, *p53*, *pRb*, *c-Jun*, *c-fos*, and multidrug resistance *MDR1*) are activated within 1 h and can increase within 30 min after stimulation with growth factors. The early growth-response gene (*Egr-1*) belongs to this group and is involved in the proliferation and cell-death pathways activated by cytokines, stress, and cytotoxic agents. RSV has been reported to induce Egr-1 expression and to increase p21 *via* an ERK1/2 pathway, which allows Egr-1 to bind to the promoter of *p21* (336). RSV appears to activate Egr-1 *via* a MAPK/ERK pathway, as opposed to a p38/SAPK2 pathway (334). Moreover, RSV activates c-Jun and c-fos and decreases c-myc activity, resulting in the loss of ornithine decarboxylase (ODC), polyamine synthesis inhibition, and increased polyamine catabolism (433). Interestingly, the myc family of transcription factors is frequently dysregulated in human cancers. Myc itself regulates gene expression through binding to enhancer box sequences (E-boxes) and by recruiting histone acetyltransferases (HATs). It has been reported that RSV affects c-myc expression and induces S-phase cell-cycle arrest, ultimately leading to apoptosis (198, 450).

Another transcription factor affected by RSV is the stress-related (protein misfolded/ER-related), small nuclear CCAAT/enhancer-binding protein-homologous protein (CHOP/GADD153). As a member of the family of the CCAAT/enhancer-binding protein (C/EBP) transcription factors, it regulates energy metabolism, cellular proliferation, differentiation, and the expression of cell phenotype–specific genes. It can form heterodimers with other members of the C/EBP family and influence gene expression by acting as a dominant-negative regulator of C/EBP, binding to one class of DNA targets, or directing CHOP-C/EBP heterodimers to other sequences. RSV induces dose-dependent apoptotic cell death in colon carcinoma cells by inducting CHOP and can be inhibited by a JNK-specific inhibitor, but not ERK, p38 MAPK, PI3K, or NF-$\kappa$B inhibitors (435). CHOP induction appears to be dependent on the transcription factor SP-1 binding to the CHOP promoter region, as shown by the luciferase reporter and SP-1 inhibitor assays.

4. RSV and redox-regulated transcription factors. Intracellular ROS exert their effect on the regulation of transcription primarily by the antioxidant responsive elements (AREs) found in the promoters of inducible oxidative- and chemical-stress genes (309). The activation of ARE enhances the antioxidant and detoxification processes seen in normal cells and may account for the chemotherapeutic resistance of cancer cells. Transcriptional activation through this enhancer appears to be mediated by basic leucine-zipper transcription factors such as Nrf-2 and small Maf proteins (108). Genomic analysis has revealed that RSV-induced cell death involves the intermediacy of redox-sensitive transcription factors. One such transcription factor affected by RSV is AP-1, which has been shown to increase in HT-29 (colon adenocarcinoma, grade II) cells when exposed to low concentrations of RSV. In contrast, luciferase assays have shown that high concentrations of RSV decrease TPA-induced AP-1 activity (200). AP-1 is formed when c-Jun, c-fos, activating transcription factor (ATF), and Jun dimerization partners (JDP) associate to form hetero- or homodimers and associate with DNA through a leucine-zipper domain to regulate the transcription of genes containing the TPA response element (TRE; 5′-TGAG/CTCA-3). Some of the phenotypic changes induced by RSV may be mediated by alterations in the AP-1 dimeric composition and by reduced intracellular ROS levels (47, 200).

RSV also may affect ARE-directed Nrf-2, which upregulates heme-oxidase 1 (HO-1) and several phase II detoxifying and antioxidant enzymes in the PC12 (rat adrenal medulla pheochromocytoma) cells (78). At the same time, a transient activation of PI3K/AKT and ERK1/2 has been observed. RSV has also been reported to activate the pregnane X receptor (PXR)-regulated CYP3A4 promoter (*i.e.,* phase I enzymes) but not phase II enzymes [*i.e.*, the electrophile responsive element (EpRE)-regulated promoters of gastrointestinal cells] (220). Quinone reductase 1 (NQO1), an enzyme involved in detoxification, is regulated by Nrf-2 and ARE and can be induced by RSV (49). For example, 25–50 $\mu M$ RSV increases the gene expression of *NQO1* in K562 leukemia cells, which peaks at 24–48 h (177). In contrast, RSV inhibits the transcription of hypoxia-inducible factor 1-alpha (*HIF-1*$\alpha$) and *VEGF* in human ovarian cancer cells A2780/CP70 and OVCAR-3 (71). This inhibition occurs by suppressing the AKT/MAPK/S6 kinase 1/S6 ribosomal protein/eukaryotic initiation factor 4E-binding protein 1 and eukaryotic initiation factor 4E. RSV had a similar repressive effect on HIF-1$\alpha$ in squamous cell carcinoma of the tongue and hepatoma cells, with the concomitant inhibition of ERK and AKT activation (451).

The most extensively studied redox-regulated transcription factor is NF-$\kappa$B, which is activated *via* canonic, noncanonic, or hybrid pathways and is composed of a class of dimeric transcription factors known as the Rel-A/NF-$\kappa$B family [c-Rel, p50 (NF-$\kappa$B1), p52 (NF-$\kappa$B2), p65 (Rel-A), and Rel-B]. NF-$\kappa$B controls the transcription of a wide number of gene products (351), and a number of dietary agents are recognized blockers of the NF-$\kappa$B signaling pathways (113). The inhibition of NF-$\kappa$B activation by RSV is associated with the antiproliferative, death-inducing, and antiangiogenic activity of RSV. RSV has been shown to block the NF-$\kappa$B activation

**FIG. 7. A simplified representation of the pathways involved in cell-cycle arrest and apoptosis induced by RSV.** None of these pathways are exclusive. (**A**) p53-p21–based pathway. (**B**) The principal cyclin:CDKs involved in each aspect of the cell cycle and where RSV can inhibit them because of regulation of their expression. (**C**) The involvement of the DNA-damage pathways ATM/ATR during the $G_1$/S and S/$G_2$ cell-cycle arrest. (**D**) The outcome of the cell-cycle arrest is dependent on the concentration used and the phenotype of the cell (*in vitro*) or the cancer stimuli (*in vivo*).

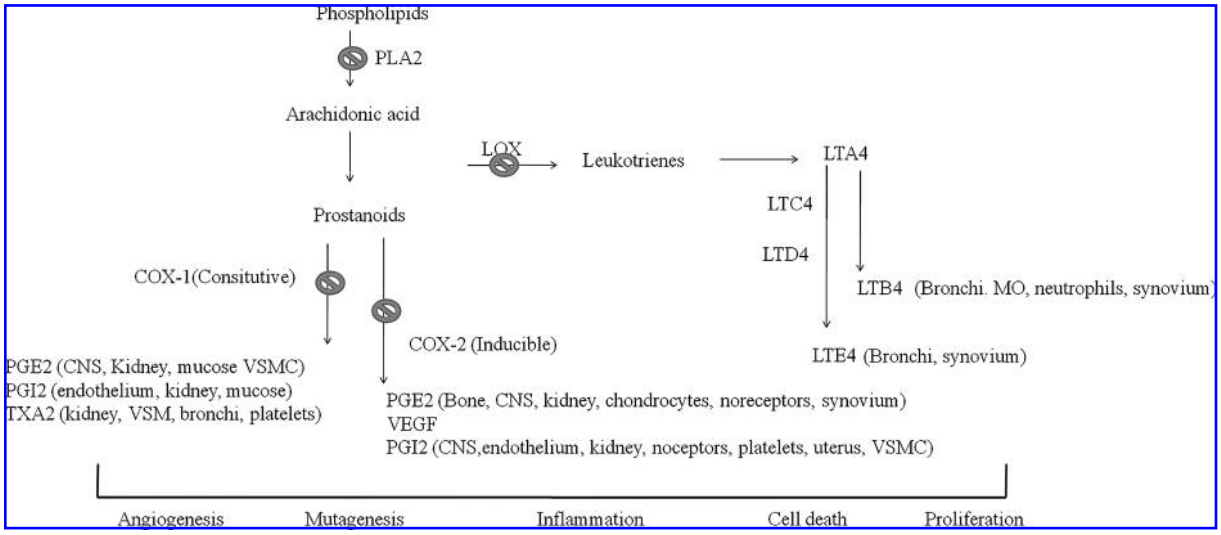



**FIG. 8. Illustration of the components of the COX and LOX pathways and the prostaglandins (PGE), thromboxanes (TRXA), and leukotrienes (LTA) and their cellular effects that RSV can inhibit.** Stop signs indicate areas RSV is known to inhibit.

induced by various carcinogens and tumor promoters, including PMA (phorbol myristate acetate), LPS, $H_2O_2$, okadaic acid, and ceramide (21, 52, 201, 267, 411, 425). Pretranslational studies in DMBA-induced tumor-bearing rats clearly show RSV's ability to decrease transcription of NF-$\kappa$B. Tumor suppression resulted from the downregulation of a variety of genes, including tumor-promoting *COX-2*, *iNOS*, matrix metalloprotease 9 (*MMP9*), and endothelial adhesion molecules (34). RSV also inhibits NF-$\kappa$B activation in endothelial cells treated with TNF-$\alpha$, but only with extended treatment (284, 314). This inhibition could be mediated by blocking TNF-$\alpha$ activation of JNK and MEK in a manner similar to that seen when RSV downregulates PMA-induced NF-$\kappa$B by blocking JNK and PKC$\delta$ activation (267). In myeloid (U-937), lymphoid (Jurkat), and epithelial (HeLa and H4) cells, RSV suppresses TNF-$\alpha$–induced phosphorylation, nuclear translocation of p65, and NF-$\kappa$B–dependent reporter gene transcription (267). Paradoxically, TNF-$\alpha$–induced apoptosis could be enhanced by RSV *via* the activation of SIRT1, which causes the deacetylation of RelA/p65 protein (444) (see Section IV.A.1. for further discussion on sirtuins). SIRT1 interacts with the NF-$\kappa$B RelA/p65 subunit and inhibits transcription by deacetylating RelA/p65 at Lys310. Thus, the RSV activation of SIRT1 may allow it to bind to the promoter region of the inhibitor of apoptosis protein 1 (cIAP-2). This, coupled with a loss of NF-$\kappa$B–regulated gene expression, leads to the sensitization of cells to TNF-$\alpha$–induced apoptosis. SIRT1 has been shown to interact physically with c-Jun, preventing its activation, and this interaction can be induced by RSV (140). Therefore, RSV can indirectly inhibit PMA- or glucose-induced AP-1–activated transcription, resulting in a decrease in MMP9 expression and mRNA.

5. RSV and lipoxygenase and cyclooxygenase. In all mammalian tissues, polyunsaturated fatty acids and triglycerides are esterified in membrane phospholipids and function primarily to maintain membrane fluidity and substrate storage. Oxidative metabolism of these fatty acids to eicosanoids (the collective name for prostaglandins, hydroxy-fatty acids, and leukotrienes) depends on the availability of free, nonesterified fatty acids. These fatty acids are substrates for three distinctively different enzymatic pathways involving lipoxygenases (LOXs), cyclooxygenases (COXs), and epoxygenases. LOXs are iron-containing dioxygenases with peroxidase activity and are involved in the synthesis of inflammatory, atherosclerotic, and carcinogenic mediators (Fig. 8) (5, 120). LOX-1 is a major receptor for oxidized low-density lipoprotein (oxLDL) and can bind damaged or apoptotic cells, activated platelets, advanced glycation end products, and pathogenic organisms. LOX-1 expression is mediated by AGN-II and endothelin-1 (ET-1), both antagonists of NO, and is associated with an increase in oxLDL levels, which in turn, enhances LOX-1 expression (289). Two isoforms of COX, COX-1 and COX-2, are found in mammalian systems (Fig. 8). COX-1 is constitutively expressed in many tissues, whereas the expression of COX-2 is regulated by mitogens, tumor promoters, cytokines, growth factors, and stress. COX, or prostaglandin H synthase, converts arachidonic acid (AA) released by membrane phospholipids into prostaglandins and has a secondary activity in the formation of hydroperoxidase (127). This hydroperoxidase activity converts PGG2 (prostaglandin G2) to PGH2 (prostaglandin H2) and indirectly generates tyrosyl radicals in the process (272). The hydroperoxidase activity may also be responsible for the bioactivation of promutagens such as phase I detoxification enzymes. The activities of COX-2, peroxisome proliferators–activated receptors-gamma (PPAR$\gamma$), and eicosanoids are linked to cancer and cardiovascular diseases, and prostaglandins are known stimulators of cell proliferation and angiogenesis and suppressors of immune surveillance.

RSV and its oxidized form can act as inhibitors of the LOX dioxygenase activity, yet maintain hydroperoxidase activity (264, 323). RSV has also been shown to inhibit the cyclooxygenase and peroxidase activity of COX-1, and the inflammatory pathways associated with COX-1 (197, 287). RSV has been shown to inactivate COX-1 over a dynamic range of peroxidase concentrations *in vivo* and therefore functions as both a mechanism-based inactivator and a co-reductant of the

COX-1 peroxidase (397). Conversely, RSV is a poor inhibitor of COX-2 hydroperoxidase activity and does inhibit COX-2 cyclooxygenase activity to a certain degree. However, COX-2 is a more robust catalyst for oxidizing RSV (396). Interestingly, RSV-mediated inhibition of COX-2 has been shown to be an effect of the phytoalexins, directly binding COX-2. This inhibitory effect was essential in preventing soft-agar colony formation of HT-29 human colon adenocarcinoma cells (454). Das *et al.* (41) proposed that RSV also possesses a novel analgesic function, as shown by using the tail-flick method, and this appears to be related to its inhibition of COX activity. This dual effect of RSV is unique as classic, nonsteroidal antiinflammatory drugs (NSAIDs) affect only COX activity (374). Indeed, RSV's structure has been used as the basis of producing more-selective COX-2 and COX-1 inhibitors (287). This is illustrated in the development of highly selective COX-1 inhibitors by using the docking-based studies of a series of bridged stilbene derivatives, based on phenyl substituted 1,2-dihydronaphthalene and 1H-indene (161).

Despite data on the ability of RSV to inhibit COX activity directly, *in vivo* evidence points to a more-indirect method *via* transcriptional regulation. Six different human uterine cancer cell lines (HeLa, Hec-1A, KLE, RL95-2, Ishikawa, and EN-1078D) were analyzed, and RSV was shown to decrease active AKT with a correlative decrease in COX-2, prostaglandin E2 (PGE2), and prostaglandin F2alpha (PGF2$\alpha$), and to increase cell death (95). In this study, the downregulation of *COX-2* transcription is related to the inhibition of NF-$\kappa$B. COX-2 has also been shown to be induced by AP-1 and ERK1/2 accumulation in the nuclei of breast cancer cells (MCF-7 and MDA-MB-231) when exposed to apoptotic concentrations of RSV (400). This accumulation allows COX-2 to associate with phosphorylated p53 and the co-activator p300, thereby decreasing p53 target gene expression. In human breast and oral epithelial cells, the PMA-induced increase in COX-2 expression and prostaglandin E2 can be prevented by RSV *via* regulating *COX-2* gene expression and direct inhibition of COX-2 (390). RSV can also inhibit COX-2 in PMA-activated mouse macrophages (274).

In MCF-7 cells, the transcription of COX-2 induced by benzo[a]pyrene and TCDD *via* AhR can be suppressed by RSV (103). The application of TPA to mouse skin induces oxidative stress shown by increased $H_2O_2$, myeloperoxidase and oxidized glutathione reductase activities, and reduced levels of glutathione, SOD, c-myc, c-fos, c-Jun, TGF-$\beta$1 and TNF-$\alpha$, and COX-2 (47, 198). This can be reversed with topical applications of RSV, which inhibits TPA-induced activation of NF-$\kappa$B and its potential targets ERK and p38 MAP kinase (230, 231). Furthermore, RSV can inhibit the TPA induction of c-fos and TGF-$\beta$1 expression, suggesting that these factors are involved in RSV-mediated inhibition of TPA-induced carcinogenesis. This indicates that RSV can function as a tumor-promotion antagonist by triggering intracellular pathways that oppose the unbalanced expression of COX-2.

## III. Modulation of DNA Damage and Cell Death by RSV

### A. RSV and DNA damage

Nucleic acid damage results in strand breaks, base-free sites, base and sugar lesions, and DNA-protein cross-links. At the correct concentrations, RSV can (a) bind directly to DNA, (b) generate genotoxic, metabolic, or inflammatory ROS, (c) modulate DNA-maintaining signals, DNA metabolism, and DNA repair, and (d) inhibit telomerase and topoisomerases. Furthermore, RSV (50 $\mu M$) has been shown to reverse ethidium bromide and acridine orange DNA intercalation, and cause strand scissons and DSB induction in circular plasmid and calf thymus DNA (391, 416). By using ultraviolet spectral analysis and Fourier-transform infrared spectroscopy, RSV has been shown to interact physically with DNA through hydrogen bonding (415). With regard to point d, RSV downregulates telomerase (131, 236, 313) and effectively inhibits topoisomerase (203, 204, 442). In a lymphoid cell line, RSV induces DNA damage, cell-cycle delays, and apoptosis in S phase (125). However, when RSV is combined with x-rays, a diminished apoptotic clearance of irradiated cells occurs, and the x-ray–induced $G_2$-phase cell-cycle arrest does not occur. RSV can inhibit the DNA damage induced in cells exposed to $H_2O_2$, and this inhibition is a direct effect of the antioxidant activity of RSV (332). Conversely, when RSV is given in combination with UV irradiation to immortalized human keratinocytes (HaCaT cells), DNA oxidative damage is observed, as indicated by the increase in 8-oxo-2′-deoxyguanosine triphosphate (8-oxo-dGTP) (79, 364).

Reactive nitrogen and oxygen species mediate changes to nucleic acids through reactions that produce useful biomarkers of nitrative stress (*e.g.*, NO-dependent formation of 8-nitroguanosine and 3-nitrotyrosine) and oxidative stress (*e.g.*, 8-oxo-dGTP and 2-hydroxy-2′-deoxyadenosine). To thwart nucleic acid oxidative damage, mammalian cells are equipped with distinct sets of enzymes. For example, the MutT homologue 1 (MTH1) protein hydrolyzes oxidized purine nucleoside triphosphates in the nucleus and prevents the misincorporation of 8-oxo-dGTP into DNA (290). In mitochondria, these oxidation and alkylation by-products are removed by the base-excision repair (BER) pathway, which involves purinic endonuclease 1 (Ape1)/redox factor-1 (Ref-1) (387). In melanoma cell lines, Ref-1 levels are higher than in normal melanocytes, and overexpression of Ref-1 can protect against cisplatin- and $H_2O_2$-induced death. This pathway keeps transcription factors in a functionally reduced state, thereby enabling the DNA-binding activity of factors such as AP-1, NF-$\kappa$B, HIF-1$\alpha$, CREB, and p53 (126, 359). Changes to Ref-1 can prevent NO damage in cardiac hypertrophy induced by RSV *in vivo* (207). The modeling of RSV and the Ref-1 pockets shows that it is likely that RSV can bind to Ref-1 and regulate its activity (443).

In response to DNA breaks, progression through the $G_1$, S, and $G_2$ phases of the cell cycle is moderated by the coordination of the ataxia/telangiectasia mutated (ATM)-checkpoint kinase 2 (ChK2)-p53 and ataxia telangiectasia and Rad3-related kinase (ATR)-ChK1 DNA damage-sensing pathways with DNA-repair mechanisms [*e.g.*, DNA-dependent protein kinase (DNA-PK) and RAD51 complexes]. Human ATM and ATR are thought to be DNA-damage sensors and belong to the PI3K gene superfamily. ATM has been implicated in the regulation of DNA double-strand breaks (DSBs) induced by ionizing radiation and other genotoxic agents. In a neuronal postmitotic model, it was recently shown that activated Cdk5 directly phosphorylates ATM at Ser794, allowing ATM to be autophosphorylated at Ser1981, and activates ATM kinase activity (404). Inhibition of the Cdk5–ATM pathway rescues cells from DNA-damage–induced neuronal cell cycle reentry and PUMA and

Bax cell death that is p53 dependent. ATR performs at least two distinct S-phase checkpoint functions: (a) the DNA-replication checkpoint, which delays mitosis in the presence of unreplicated DNA; and (b) the prevention of replication fork collapse and DNA-strand breakage when DNA replication is transiently inhibited. In the absence of functional p53, DNA-damaging agents can activate an alternative pathway that uses p38 MAPK and MK2 (MAPKAPK2), which occurs downstream of ATR/ATM, depending on the stimulus (338). Recent data showed that, should ATR/ATM cause prolonged phosphorylation of H2AX on Tyr142 due to the absence of EY1, this will cause apoptosis through a JNK1-Fe65 and Mre11–Rad50–Nbs1 (MRN)-MRN complex. However, should EY1 dephosphorylate H2AX, JNK1-Fe65 is not recruited, and the cell undergoes a survival and repair to its DNA (88).

RSV influences the ATM/ATR pathways to induce S-phase arrest (Fig. 7) (412). The underlying mechanism is triggered by the activation of the checkpoint kinases, Chk1 and Chk2, and the phosphorylation of cdc2 at Tyr15 coupled with amplification of phospho-H2AX (Ser139). In tumor cells, the ability of low levels of RSV to induce a prooxidant state (169) may be related to an increased level of mitochondrial ROS, which induces senescence (145). p38 MAPK, p53 and p21, and the ATM kinase pathway are implicated in this process, as RSV initiates replication stress. Similarly, the tumor-suppressor *BRCA* genes and their related family members are involved in DNA damage repair and are strongly implicated in hereditary breast cancer (122). Interestingly, at relatively low micromolar ranges, RSV can increase the expression of BRCA1 (breast cancer type 1, early onset) and BRCA2 (breast cancer type 2 susceptibility protein) in breast cancer cell lines (MCF-7, HBL-100, and MDA-MB 231) (137). It has been shown that SIRT1-BRCA1 is involved in the modulation of DNA damage. In a $Sirt1^{+/-}$; $p53^{+/-}$ background, multiple-tissue tumors occur in mice. The occurrence of these tumors can be reduced by RSV treatment through the ability of RSV to activate SIRT1 (427).

A second study shed light on the regulation of survivin *via* BRCA-1 and SIRT1 (428). The study demonstrated that BRCA-1 binds to the *SIRT1* promoter, allowing SIRT1 to suppress the expression of survivin. This regulation appears to be lost in BRCA-1–mutated breast tumors. On the addition of RSV, SIRT1 is reactivated, thereby bypassing the need for BRCA1, and decreasing survivin expression, leading to a decrease in cell proliferation. RSV has also been reported to inhibit homologous recombination (HR) and nonhomologous end joining (NHEJ) in a manner independent of its known growth- and death-regulatory functions, but dependent on ATM/ATR, p53, and Nbs1 (Nijmegen breakage syndrome) (143).

Another feature of RSV is its ability to bind metal. In the presence of copper [Cu(II)], RSV induces a high incidence of micronuclei, sister chromatid exchange, and DNA-cleaving activity (28, 132). Metal-bound RSV primarily induces point mutations, whereas RSV devoid of metal predominantly induces deletions of guanine bases (7). According to research, as copper is bound to guanine bases in chromatin, the endogenous copper can interact with RSV, resulting in DNA cleavage at the binding site. Through the use of analogues with different nuclease capabilities, a mechanism involving the formation of a copper–peroxide complex under prooxidant conditions has been postulated (133).

### *B. Cell death*

Based on biochemical and morphologic hallmarks, cell death has been largely classified as either apoptotic, necrotic, or autophagic. In recent years, novel cell-death mechanisms such as necroptosis and paraptosis have also been described in a variety of model systems (139, 150). Collectively, these death processes are essential for tissue homeostasis and for maintaining normal embryonic development.

Apoptosis is predominantly a highly controlled protease caspase-dependent cell death mechanism. The apoptotic machinery functions primarily through an extrinsic (death-receptor) pathway, or an intrinsic (mitochondrial) pathway, or in some instances, a combination of both. The apoptosis signaling downstream of death-receptor stimulation (the extrinsic pathway) could follow one of two signaling pathways, as was first reported downstream of the CD95 (Apo1/Fas) receptor ligation. Stimulation of the death receptor triggers receptor oligomerization and recruitment of the adaptor protein FADD (Fas-associated death domain), which binds to the cytoplasmic aspect of the death receptor *via* death domain (DD)-DD interaction, and recruits procaspase-8 to this assembly *via* its death-effector domain (DED). This membrane proximal complex is termed the death-initiating signaling complex (DISC), which, once formed, results in the autoprocessing (activation) of procaspase-8 to the active caspase-8. In the first scenario, DISC assembly and the resultant activation of caspase-8 is strong and robust and drives downstream caspase activation, resulting in the ultimate activation of the executioner caspase-3 and DNA fragmentation. This type of apoptotic execution is referred to as extrinsic apoptosis, and cells in which receptor ligation follows this signaling pathway are termed type 1 cells. In the alternate pathway, death-receptor stimulation does not affect a strong enough assembly of the DISC components, and the activation of caspase-8 is not strong enough to engage downstream caspases directly. However, the low level of caspase-8 activation cleaves the proapoptotic Bcl-2 family protein Bid, which then signals the translocation of Bax to the mitochondria, thereby resulting in the egress of apoptosis amplification factors from the mitochondria to the cytosol, such as cytochrome *c* (cyt.c), apoptosis-inducing factor (AIF), Smac/Diablo, and others. These amplification factors facilitate the assembly of another complex, the apoptosome, comprising the protein apoptosis protease-activating factor-1 (Apaf-1), procaspase-9, and cytochrome *c*, and triggers activation of caspase 9, which then drives the executioner caspase-3. By dint of its reliance on mitochondria-derived prodeath factors, this pathway is referred to as the intrinsic or mitochondria-dependent apoptosis pathway, and the cells in which this pathway is predominant are termed type II cells (353). In line with this, overexpression of the apoptosis-inhibitory protein Bcl-2 has minimal effect on the extrinsic pathway of apoptosis, but completely blocks intrinsic death execution (173). The intrinsic pathway of apoptosis is also the favored signaling network triggered on exposure of cells to DNA-damaging agents and UV radiation, which becomes important in the clinical setting, where cancer cells exhibit apoptosis resistance because of the overexpression of Bcl-2/$Bcl_{xL}$. A number of studies have demonstrated the involvement of both the extrinsic and the intrinsic pathways in RSV-induced apoptosis in cancer cells *via* its ability to regulated free radical production as well as protein expression and activity. The control of cell death is tightly linked to the redox

environment by the modulation of a number of factors, including receptor engagement, and anti- and proapoptotic protein expression and activity (*e.g.*, MAPK/apoptosis signal–regulating kinase 1 (ASK1)/Trx), mitochondrial stability, and cellular energy status). By modulating the redox environment, a resistant type 2 cell line can be switched to a sensitive type I cell line (319). Therefore, systems that produce free radicals will have a prominent influence on cell death and survival, either directly or indirectly (318). This is very nicely illustrated by the finding that TNF-α–induced necrosis triggers both the activation of NOX-1 and the redox regulation of c-FLIP (FLAME/FLICE, Casper, MRIT, CLARP, and Usurpin) (419). GPX4-LOX has recently been added to the list of pathways that can modulate cell death in a redox-dependent manner *via* its ability to induce regulate stress-apoptosis–inducing factor (AIF)-mediated cell death in mice (361).

In contrast to apoptosis, necrosis is largely a caspase-independent process, resulting in excessive cell damage, the rupture of cell membranes, the release of cellular contents, and a large and complex immune response. Although originally thought to be an unordered process, recent evidence suggests that necrosis can be systematic. It is important to note that other cell-death systems exist, such as those that use the proteins granzyme B and perforin. Both of these are secreted proteins that induce death through their activity at transmembrane pores and through the activation of caspases and the calcium-activated proteases calpain and cathepsin. Although RSV can inhibit cathepsin D, which is an aspartyl lysosomal protease, little else is known about the effect of RSV on these proteins (55, 176, 410, 424).

The last form of cell death is related to autophagy, which is a form of cellular self-regulation that renders the cell in a state of "limbo" [*i.e*, the cell does not die, but instead digests dysfunctional organelles during senescence, or cell death occurs over time (151)].

1. RSV and membrane-mediated apoptosis signaling. The plasma membrane contains a multitude of death sensors and instigators, including receptors, lipids, secreted proteins, and radical species. It is well established that membranes are composed of specific lipids that can be involved in signaling (as exemplified by the PI3K/AKT-PTEN pathway, phosphatidylserine (PS), and mitochondrial cardiolipid) or can regulate spatiotemporal orientation and hence the activity of receptors via lipid rafts. This is illustrated by the T cell–receptor ability to activate caspase-8 and trigger either proliferation or death depending on its location.

Regarding cell death, the sphingolipid ceramide appears to be the most important lipid. Ceramide is a mediator of intracellular signals and is normally present in membranes as a complex of sphingomyelin or gangliosides. During conditions of stress and aging, there is increased *de novo* synthesis of ceramide and the release of complex sphingolipids by hydrolysis occurs (268). RSV promotes the intracellular accumulation of ceramide in breast and prostate cancer cells (111, 355, 356). It also enhances the *de novo* synthesis of this sphingolipid by increasing the activity of the rate-limiting enzyme. This process can be rescued by ceramide-enzyme blockers (111, 355) and ornithine decarboxylase activity in colon cancer cells (414). This identifies an important checkpoint in the actions of RSV, suggesting that the production of ceramide may be a common determinant that drives cells irreversibly toward death. Another mechanism by which RSV functions at the membrane is through the modulation of the death receptors, including those of the TNF-receptor superfamily and the Toll-like receptors (TLRs). RSV can induce CD95-dependent cell death by upregulating the surface expression of CD95 ligand (CD95L/CD178) in HL-60 cells and T47D cells, but not in normal PBMCs (87). It was subsequently shown that RSV induces the redistribution of CD95 into lipid rafts, resulting in the sensitization of these tumor cells to death receptor–mediated apoptosis (107). This finding has been expanded to encompass other receptors, such as the death receptor 4, 5 (DR4, 5) of TRAIL.

2. RSV and mitochondria-dependent intrinsic apoptosis. RSV has been shown to have a direct effect on mitochondria through its ability to induce the opening of the mitochondrial transmembrane permeability pore (MTP) by decreasing the mitochondrial threshold level of $Ca^{2+}$ (349). Furthermore, RSV inhibits the $F_1F_O$-ATPase pump by targeting the $F_1$ catalytic domain (38, 147). Another channel protein targeted by RSV is the sulfonylurea receptor-1 (SUR) (20, 160), which constitutes the regulatory subunits of $K_{ATP}$ channels that are activated in specific models of apoptosis (66, 352). By using the L6 rat skeletal muscle cells, RSV was shown to decrease the expression of the $Na^+/H^+$ exchanger-1 (NHE-1), which is involved in pH regulation *via* activation of caspase-3 (initial deregulation) and caspase-6 (late/committed deregulation) in an iron- and $H_2O_2$-dependent manner (202). However, the specific mechanism by which RSV activates caspases, either directly or indirectly, is still unclear. This is exemplified in the HCTT16 (colon cancer) cell line, which can undergo p53- and Bax-independent cell death in response to RSV *via* activation of caspase-6 and lamin A cleavage (242). In addition, caspase-2 activation is thought to be the initial event of RSV-induced apoptosis in these cells, triggering Bax/Bak and mitochondria-mediated events that are independent of NF-κB and caspase-8 (281). However, translocation of Bid requires the presence of both caspase-2 and caspase-8.

As previously documented, RSV exerts an anticancer effect by causing apoptosis in the wake of cell-cycle arrest in a range of cell lines (Fig. 7). This death appears to be due to a number of different mechanisms, including increased caspase activity, cell-cycle arrest in the $G_1$ phase, inhibition of cell-cycle progression from the S to $G_2$ phase, decreased protein levels of cyclin D1, Cdk4, Bcl-2, and Bcl-$X_L$, increased Bax levels and the induction of the Cdk-inhibitor p21. DU145 cells undergo apoptotic cell-cycle arrest in response to RSV via negatively affecting cyclins and Cdks and at the same time activating pro-apoptotic signaling such as Bax activation and enzymatic induction of caspases 3 and 9. However, the levels of anti-apoptotic elements such as Bcl-2 and Bcl-$X_L$ remain unaffected (219). This is an important factor, as resistance to apoptosis depends on the intracellular balance of proapoptotic and prosurvival factors, such as the members of the Bcl-2 family, and the inhibitors of apoptosis (IAPs) (135). RSV is able to regulate the protein expression of IAP proteins such as survivin, a bifunctional protein involved in mitosis as well as in cell death (365). Survivin is required for the $G_1$/S transition phase and remains high during mitosis; it participates in chromosome assembly at the mitotic spindle. Survivin expression is regulated by the formation or dissociation of different Rb/E2F complexes and can be switched off by an

increase in p53/p21 complexes. The role of survivin in preventing apoptosis is somewhat debated, but it appears to inhibit caspase activation and cause mitochondrial dysfunction in events that appear to be dictated by its compartmentalization (14). In tumor cells, it has been found to be antiapoptotic in the mitochondria. However, if survivin is relocated to the cytoplasm (where it resides in normal cells), it is proapoptotic. RSV appears to decrease survivin levels by enhancing its degradation and reducing its transcription. These actions correspond with the observed decrease in proliferation and sensitization to chemotherapy (46, 166). RSV sensitizes cells to TRAIL-induced apoptosis *via* a $G_1$ cell-cycle arrest and survivin depletion. However, it fails to sensitize cells that have abnormally high levels of Bcl-2 or fas-associated death domain–dominant negative (FADD-DN), despite undergoing cell-cycle arrest and survivin depletion (134). This can be explained by an underlying inhibition of the release of the proapoptotic proteins cytc and Smac/DIABLO to the cytosol from the mitochondria. RSV has been shown to reverse clusterin's inhibition of activated Bax in docetaxel-resistant PC3-DR and DU145-DR prostate cell lines, thereby sensitizing them to TRAIL-induced cell death (348). This study showed that the reversal of clusterin activity was due to inhibition of Src and Jak kinases by RSV, thereby leading to the loss of STAT1, which regulates clusterin expression. This could also explain the mechanism by which RSV induces sensitization to the chemotherapeutic agent docetaxel. Thus, RSV is thought to be a potent sensitizer for TNF-based treatments of certain types of cancer through the depletion of survivin.

A critical component of DNA damage–induced cell death is the cleavage of the DNA-repair enzyme poly (ADP-ribose) (PAR) polymerase-1 (PARP-1), a substrate of the executioner caspase-3 (163). PARP-1 catalyzes the interaction of $NAD^+$ with polymers of poly (ADP-ribose) (PAR) (Fig. 6). Of note, PAR translocates from the nucleus to the mitochondria, where it triggers the release of AIF (168, 222). AIF then translocates to the nucleus and induces a nuclear-based death. Mitochondria contain (ADP-ribose) glycohydrolase (PARG), which can degrade PAR (296). Additionally, an increase in nuclear $NAD^+$ triggers the opening of the MPT, thereby establishing a feedback loop in response to excessive PARP activity that leads to the induction of cell death. Reduced PARP-1 activity has been shown to be a consequence of RSV-activating SIRT1. If SIRT1 is absent, PAR synthesis increases, which can lead to AIF-mediated cell death (222). In normal cells, the mitochondrial ATP is directly connected to nuclear PARP-1 *via* a specific adenylate kinase enzymatic path. The nuclear target of PARP-1 is topoisomerase I, and this interaction has been identified as a critical "switch-point." The mitochondrial-nuclear PARP-1 pathway has been specifically described in transformed cells (229). How RSV might affect PARP-1 *via* an ATP/$NAD^+$-dependent system is still unknown.

3. RSV and autophagy. The term autophagy was coined to describe a process that shared some features of lysosomal degradation, but which was also distinct. Autophagy is now divided into macroautophagy, mitoautophagy, and chaperon-mediated autophagy. Autophagy appears to work in the capacity of cellular housekeeping through modulation of nutrition sensing, developmental/programmed cell death, endoplasmic reticulum stress, and immunity in various pathologies, such as neurodegeneration, cancer, viral infections, muscular disorders, and aging.

The first molecular link between autophagy and cancer was seen with the discovery of the beclin-1 gene (the mammalian homologue of the yeast Atg6), which has been shown to interact with Bcl-2. Since then, a number of reports mentioned beclin-1 interacting with proteins to regulate the autophagy process. The autophagy-related proteins (ATG), the AKT-mTOR pathway, and, recently, JNK have all been identified as autophagic mediators. It is therefore not surprising to find reports detailing autophagy as a redox-sensitive mechanism, and it is plausible that autophagy is a regulator of stress-related events (260, 357, 358). It is also intriguing to note that macroautophagy declines with age and that lifespan extension is dependent on maintaining active autophagy. The molecular mechanism behind this may be related to the longevity proteins, sirtuins, that bind to and deacetylate the autophagic proteins Atg 5, 7 (which form the Atg12 conjugation systems) and Atg 8 (LC3), thereby allowing the autophagic process to occur and clear damaged organelles. In the process of delineating autophagy from cell death versus cell survival, it has become apparent that it is the survival-based aspects of autophagy that cancer manipulates to maintain cell survival. By switching this autophagy to a death-inducing autophagy, many chemotherapeutic strategies can trigger cell death in these cells. Interestingly, the activity of PI3/AKT and PTEN in the context of autophagy is becoming important, as seen by the ability to trigger autophagy-dependent cell death by using lysosomotropic agents in PTEN-null cancer (104).

RSV can reduce phosphorylated-AKT and mTOR, which, in turn, leads to the regulation of glycolysis (Fig. 4) (227). RSV can also work in a manner similar to the overexpression of SIRT1 in cells. This induces proliferation *via* increased ERK and S6K1 phosphorylation, which, in SIRT1-overexpressing cells, is mediated by an mTOR-based pathway (182). RSV is reported to be able to suppress the mitogen-induced phosphorylation of S6K (a target of mTOR) and subsequent rat aortic smooth muscle cell hypertrophy (157). Additionally, RSV has the ability to activate AMP-activated protein kinase (AMPK) and stimulate mitochondrial biogenesis in an AMPK-LKB1– dependent/SIRT1-independent manner (99). RSV is also reported to activate AMPK *via* the generation of ROS in HT-29 cells, and this study showed that the combination of RSV and etoposide induced AMPK activation and apoptosis and inhibited cell growth (185). RSV can increases glucose uptake in C2C12 (mouse myoblast) myotubes cells by activating AMPK independent of the PI3K kinase signal pathway (310). Another part of the pathway has been shown in which AMPK can regulate glucose metabolism through increasing $NAD^+$ levels, which, in turn, increases SIRT1 activity, resulting in the deacetylation and modulation of the activity of downstream SIRT1 targets, including the PGC-1$\alpha$, FOXO1, and FOXO3a transcription factors (70). This pathway is likely to help explain the beneficial effects of RSV in diabetes and is in line with the use of metformin activation of AMPK being beneficial for the treatment of type 2 diabetes.

Considering that RSV modulates the expression and function of several proteins, together with the cellular redox environment, it is plausible that its ability to modulate autophagy may help explain the duality of RSV, as the mTOR

pathway regulates different subsets of genes in response to oxidative stress and nutritional deprivation *via* raptor (rapamycin sensitive *via* FK506-binding protein) or rictor (rapamycin insensitive), as illustrated in Fig. 4 (56). An oxidative environment activates the raptor-mTOR interaction, and one way this can occur is through mitochondrial metabolism in a nutrient-rich environment. These conditions would allow the activation of the nutrient-sensing raptor-mTOR pathway by modifying a redox-sensitive mechanism on raptor-mTOR. Reevaluation of the cell death seen when RSV is added to human ovarian carcinoma cell lines has shown that, although many of the molecular markers of apoptosis where present (cyt c release, apoptosome complex formation, and caspase activation), microscopic evaluation of the morphology and ultrastructure indicated that the process resembles autophagy (305). RSV can also induce autophagy-dependent cell death in DLD1 (human colorectal cancer) cells (409). Another study with the caspase-3–deficient MCF-7 cells showed that RSV induces noncanonic autophagy in a beclin-1–independent manner, which has significant implications, as the tumor-suppressor beclin-1 is downregulated in a number of tumor cells (354). RSV has also been shown to induce autophagy-selective degradation of proteins, such as the proline-rich, glutamic acid–rich, and leucine-rich protein-1 (PELP1; which is reported to be able to bind to ER$\alpha/\beta$Rb and recruit histone deacetylase) (302).

## IV. The Beneficial Effects of RSV in Physiological Models

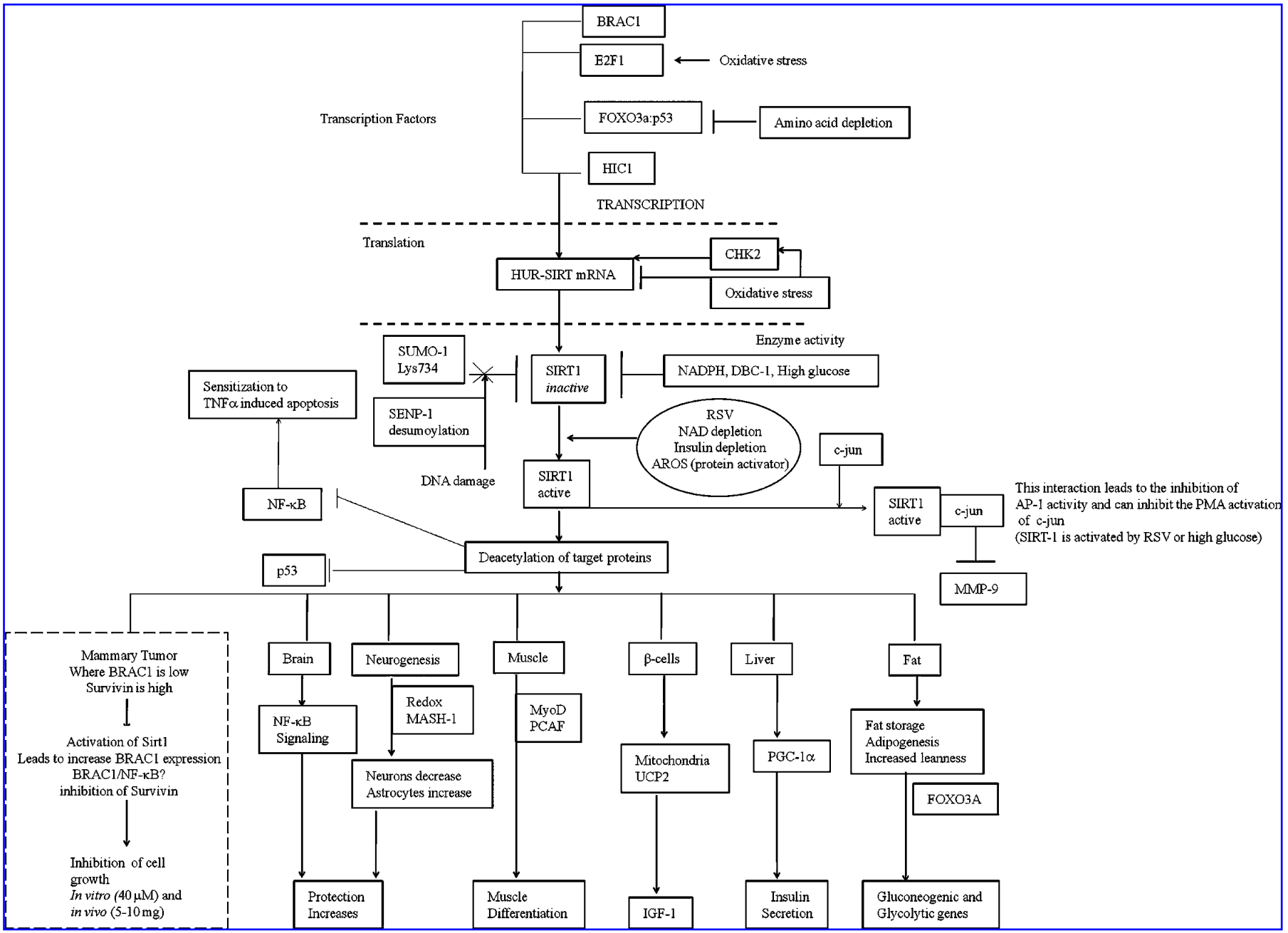
RSV influences a number of health-related processes, and its small molecular structure makes it a potentially important lead compound for the development of targeted analogues or a dietary supplement. A few examples of the effects of RSV on human health are discussed later.

### *A. The influence of RSV on lifespan and metabolism through sirtuins*

The finding that caloric restriction (CR) while maintaining nutrition levels could extend the lifespan of an organism has led to investigation into the mechanisms behind this, including a decrease in oxidative damage, altered glucose levels, an increase in insulin sensitivity, neuroendocrine modulation, an increase in stress responsiveness, hormesis, and the modulation of gene expression. The influence of a group of proteins know as silent information regulator 2 (Sir2) proteins, or sirtuins, in the regulation of lifespan came to light with the use of the model *S. cerevisiae* in which Sir4 was initially thought to be the key player, but it was later shown that Sir2 could extend the lifespan of this organism (153, 188, 208, 216, 256, 406). Sirtuins play an important role in glucose metabolism, AMPK regulation, insulin secretion, skeletal muscle, and fatty acids. One of the major breakthroughs in the field was the discovery that RSV activates Sir2 (the yeast homologue of human SIRT1) (175, 256). Subsequent studies in both animal and human models indicate that RSV reduces the $K_m$ without affecting the overall turnover rate of SIRT1, which is regulated at a number of levels in the cell (Figs. 4 and 9). However, it should also be noted that the reproduction of *in vivo* SIRT1 activity by RSV is not as easily accomplished as that *in vitro*, suggesting that RSV may actually act on Sirt1 in an indirect manner (58).

Sirtuins (abbreviated to SIRTs in humans and Sirts in mice) are a family of $NAD^+$-dependent histone deacetylases (HDACs) that are different from the classic $Zn^+$-dependent HDACs. Although their principal action is to deacetylate proteins, some SIRTs are efficient depropionylators and debutyrators (Hst2, Sirt1, Sirt2, and Sirt3). It also is important to note that Sirt4-7 deacetylation activity is relatively weak; however, they are efficient in propionylation and butrylation. Moreover, Sirt4 and 6 have been reported to possess ADP-ribosyltransferase activity (376). In mammals, seven SIRTS are found, and their functions are related to their principal cellular localization: the nucleus (SIRT1, SIRT2, SIRT6,) and nucleolus (SIRT7), the cytoplasm (SIRT1 and SIRT2), and the mitochondria (SIRT3, SIRT4, and SIRT5). SIRTs are activated in response to CR, oxidative stress, and other physiologic events (Fig. 9). They may prolong the organism's lifespan though modulation of gene expression and mitochondrial function, mobilization of white fat (an energy depot, as opposed to brown fat, a heat depot) in a process that is partially related to the activity of p66Shc (the 66-kDa isoform of the growth-factor adapter Shc) and the merging of DNA repair with the overall energy balance (154, 158, 321). In response to stress, the mitochondria-based SIRT3 is able to deacetylate Ku70, which allows it to interact with active Bax and inhibit the death function of Bax (394). This suggests that SIRTs are part of a redox-based "survival-acetylation switch." RSV enhances SIRT1 chromatin association in the *cIAP-2* promoter region in a manner that is dependent on the loss of NF-$\kappa$B (RelA/p65)-regulated gene expression (444). This sensitizes cells to TNF-$\alpha$–induced apoptosis, even though SIRT1 protects against p53-induced apoptosis. This protective effect is related to the ability of SIRT to bind and deacetylate p53 and p73, thereby repressing the expression of their target genes (91, 399). Data suggest that under normal conditions, SIRT6 acts at the chromatin, where it interacts with RelA and deacetylates histone H3 Lys9 at NF-$\kappa$B target-gene promoters (214). A deficiency in SIRT6 can lead to premature aging because of the loss of this mechanism.

The SIRT family exerts some of its regulation *via* the FOXO transcription factors, which act as cell-proliferation inhibitors and apoptotic cell-death promoters, as well as protecting cells from DNA damage and oxidative stress. SIRT1 has also been implicated in stem cells and neurogenesis (33, 251, 286, 329). The SIRT family also regulates the deacetylation of FOXO-4, which serves to enhance the transcriptional activation of FOXO-4 with the transcriptional coactivator p300. The activity of FOXO-4 can be suppressed or enhanced by the SIRT1 inhibitor nicotinamide or its activator RSV (221). This activation of SIRT1 is related to the increase in the p53 stress-response protein GADD45 (growth arrest and DNA-damage gene). The amino acid motif LXXLL in FOXO1 has been shown to be the binding site for SIRT1, and RSV can induce this protein–protein interaction. This binding regulates the sensitivity of insulin *versus* gluconeogenesis in cells, as FOXO-1 is a negative regulator of the insulin/IGF pathway and induces the expression of Igfbp and glucose-6-phosphatase (291). It is thought that the protein levels of FOXO-1 are binary and are regulated by AKT-mediated phosphorylation and S-phase kinase-associated protein 2 (Skp2/p45)-mediated ubiquitination (180). Given that the AKT and Skp2 proteins are highly activated in human cancers because of the loss


BRAC1
E2F1
Oxidative stress
Transcription Factors
FOXO3a:p53
Amino acid depletion
HIC1
TRANSCRIPTION
Translation
CHK2
HUR-SIRT mRNA
Oxidative stress
Enzyme activity
SUMO-1
Lys734
SIRT1
inactive
NADPH, DBC-1, High glucose
Sensitization to
TNFα induced apoptosis
SENP-1
desumoylation
RSV
NAD depletion
Insulin depletion
AROS (protein activator)
c-jun
DNA damage
SIRT1
active
NF-κB
SIRT1
active
c-jun
This interaction leads to the inhibition of
AP-1 activity and can inhibit the PMA activation
of c-jun
(SIRT-1 is activated by RSV or high glucose)
Deacetylation of target proteins
p53
MMP-9
Mammary Tumor
Where BRAC1 is low
Survivin is high
Activation of Sirt1
Leads to increase BRAC1 expression
BRAC1/NF-κB?
inhibition of Survivin
Inhibition of cell
growth
In vitro (40 μM) and
in vivo (5-10 mg)
Brain
Neurogenesis
Muscle
β-cells
Liver
Fat
NF-κB
Signaling
Redox
MASH-1
MyoD
PCAF
Mitochondria
UCP2
PGC-1α
Fat storage
Adipogenesis
Increased leanness
FOXO3A
Neurons decrease
Astrocytes increase
Protection
Increases
Muscle
Differentiation
IGF-1
Insulin
Secretion
Gluconeogenic and
Glycolytic genes

of PTEN, the deregulation of the FOXO proteins appears to be a promising cancer target (181). Furthermore, FOXO3a, together with p53 and SIRT1, forms a "nutrient-sensing pathway" that has been shown to be activated by RSV (295). RSV can also regulate the "fasting-inducible" switch [p300 SIRT1, CREB-regulated transcription coactivator 2 (CRTC2/TORC2), and FOXO1] (258). This study showed how two gluconeogenic regulators interact during fasting to maintain the energy balance.

The physiologic effects of RSV appear to be related to its ability to regulate nutrition and longevity genes. Evidence indicates that RSV reduces fat accumulation in mice *via* SIRT1 repression of PPAR$\gamma$ and its target genes (321). RSV is also able to improve glucose homeostasis in mammals by stimulating the SIRT1-mediated deacetylation of the transcriptional coactivator peroxisome proliferator–activated receptor-gamma (PPAR$\gamma$) coactivator-1$\alpha$ (PGC-1$\alpha$) (36). With mouse models, RSV was shown to increase mitochondrial mass and function, which positively affects their muscle-fiber performance (234). These effects contributed to a decrease in PGC-1$\alpha$ acetylation and an increase in PGC-1$\alpha$ activity *via* the SIRT1 pathway, thereby protecting against obesity and insulin resistance.

### B. RSV and osteoporosis and diabetes

Two other disease areas in which RSV may have potential benefits are osteoporosis and obesity/diabetes. RSV treatment was shown to protect bones because of the induction of bone morphogenetic protein-2 through ER activation *via* a FOXO1a-Src kinase pathway (388). Other mechanisms of bone protection exist, as shown by Dai *et al.* (92), in which RSV can stimulate proliferation and osteoblastic differentiation through ER-dependent ERK1/2 activation. RSV can also induce mesenchymal stem cells to differentiate into osteoblasts in a bone-inducing medium containing adipocytes (31). This appears to be related to the activity of SIRT1, which reduces adipocyte formation and promotes osteoblast differentiation *via* the inhibition of PPAR$\gamma$2. In adipocytes exposed to free fatty acids, RSV triggers FOXO1 nuclear translocation and increase in FOXO1 protein levels, a decrease in the generation of ROS, and a partial reversal of the proinflammatory adipokine pattern (389).

RSV also modulates glucose uptake, a process that is important in diabetes. RSV can produce a dose-dependent hypoglycemic effect and an increase in insulin levels in streptozotocin-induced (STZ) diabetic rats (83). STZ-diabetic rats treated with RSV (3 mg/kg, PO) for 7 days exhibited normalized hepatic phosphoenolpyruvate carboxykinase (PEPCK) expression and increased GLUT-4 expression in the soleus muscle. RSV inhibits insulin secretion by (a) inhibiting the amplifying pathway of insulin secretion, (b) exerting insulin-suppressive effects independent of its estrogenic/antiestrogenic activity, (c) shifting islet glucose metabolism from mitochondrial oxidation to anaerobic, (d) failing to abrogate insulin release promoted without metabolic events, and (e) not suppressing hormone secretion as a result of the direct inhibition of $Ca^{2+}$ influx through voltage-dependent $Ca^{2+}$ channels (398). RSV treatment (3 mg/kg, IP) increased insulin secretion and reduced plasma glucose levels in normal rats, but not in STZ-diabetic rats, within the initial 60 min (80). This appears to be due to the inhibition of I(KATP) and I(KV), which can contribute to plasma glucose-reducing effects in normal rats. RSV activates SIRT1 and thereby increases insulin sensitivity both *in vitro* and *in vivo* (2.5 mg/kg/day) by repressing protein tyrosine phosphatase-1B (PTP1B) transcription at the chromatin level (393). Choudhury *et al.* (422) also demonstrated the ability of RSV to target activated PTP1B, which inhibit the PDGF receptor in a dose-dependent manner ($IC_{50}$, 10 $\mu M$). By using the Zucker spontaneous genetic obesity model system (in which rats develop type 2 diabetes and abnormal heart function), Tosaki *et al.* (244) showed that RSV treatment (5 mg/kg) could confer protection in glucose-fed Zucker rats. In this study, insulin levels remained the same as those of the control group, whereas the glucose levels and body weight decreased (244). RSV has also been shown to mimic CR at the genomic and proteomic levels and to prevent the adverse effects seen with a high-fat diet (35). This has spurred interest in developing small molecules that activate SIRT1 for treating age-related disease such as type 2 diabetes. Several promising candidates have already been developed as potential treatments (278).

### C. RSV and neurologic disorders

Emerging literature indicates that the aging process and neurologic diseases such as Alzheimer's disease (AD) are intrinsically linked and that modulation by both CR regimens and CR mimetics, such an RSV, can abrogate these diseases (257). Epidemiologic studies have paved the way toward the hypothesis that moderate wine consumption reduces the risk of AD (246). Amyloid-producing cell lines respond to RSV treatment by reducing the levels of secreted and intracellular amyloid-$\beta$ peptides through promoting their clearing by proteosomal degradation (269). In AD, NO release is increased, and an apparent inhibition of AA production occurs by upregulation of cytosolic phospholipase A2 (cPLA2) and AA-CoA acetyltransferase (74). One study showed that RSV (100 $\mu M$) prevents NO formation and induces AA production, highlighting the RSV potential in the management of AD. It should also be noted that SIRTs are implicated in the protection against AD as well as amyotrophic lateral sclerosis (217). In addition, in wallerian degeneration slow mice, RSV has been shown to be protective against axotomy neuronal degeneration by enhancing SIRT2-mediated tubulin deacetylation (395) and increasing NAD synthesis (16). Therefore, because RSV can activate SIRTs, it may have therapeutic potential in the management of AD and other neurologic diseases; however, further studies are needed.

The antioxidant qualities of RSV may also be useful in dampening the excitatory neurotransmitter toxicity associated

**FIG. 9. The regulation of sirtuins occurs at three levels; transcription, translation, and protein activity/degradation.** SIRTs are kept inactive by sumoylation as well as binding to inhibitor proteins such as DBC-1, whereas their activity can be enhanced by AROS and desumoylation on receipt of the correct stimuli. SIRTs can induce tissue-specific responses through the activation of the transcription factors.

with glutamate and in dopaminergic neurodegenerative disorders such as Parkinson's disease (PD). This was illustrated by pretreating SH-SY5Y (human neuroblastoma) cells with RSV, which resulted in an inhibition of dopamine cytotoxicity (241). The Parkinson's model of inhibiting the mitochondrial complex I by 1-methyl-4-phenylpyridinium ($MPP^+$) or serum potassium withdrawal has also been used to study the effects of RSV (15). In this model, RSV inhibited the cell death seen with $MPP^+$. This inhibition was independent of the stimulation of SIRT1 but dependent on the antioxidant abilities of RSV. RSV is also thought to have beneficial effects in Huntington's disease (HD), which is a fatal disorder caused by polyglutamine repeat expansion in the Huntington's gene. In this regard, the antioxidant ability of RSV (5 and 10 mg/kg given orally), coupled with its COX-1 inhibitory activity, significantly reverses the damage induced by 3-nitropropionic acid, a mitochondrial citric acid–cycle inhibitor that mimics HD effects on the mitochondria and can cause motor deterioration *in vivo* similar to that of HD (228). In HD mouse models, the RSV induction of SIRT1 protects neurons against polyglutamine toxicity (312). However, the exact bioenergetics dysfunction is still unclear. With an inducible yeast model expressing a human mutant polyglutamine tract of the 103Q Huntington's fragment, RSV has been shown to correct the abnormal cellular respiration caused by alteration in the function and amount of mitochondrial respiratory chain complex, and by an increase in ROS (377). Along similar lines, the Huntington's mouse model has reduced food intake and increased energy expenditure with dysfunctional mitochondria (showing a reduced rate of oxygen consumption) in brown adipose tissue (432). Although sympathetic stimulation of PGC-1α was intact in these mice, the induction of uncoupling protein-1 (UCP-1) was reduced. With cultured cells, it was shown that the expression of the Huntington's mutation suppressed UCP-1 promoter activity. This could be reversed on expression of PGC-1α. PGC-1α is a known regulator of mitochondrial function, and in Huntington's mice, the striatal neurons expressing exogenous PGC-1α are resistant to 3-nitropropionic acid treatment (432). It is therefore hypothesized that an altered PGC-1α function may provide a link between transcription deregulation and mitochondrial dysfunction in HD. It was recently reported that PGC-1α can stimulate mitochondrial electron transport while suppressing ROS levels (383). This would provide a clear mechanism whereby tissues, such as skeletal muscle and brown fat, can augment mitochondrial metabolism in response to external conditions without causing self-inflicted oxidative damage. It is at present unclear how RSV may directly or indirectly affect this system; however, a recent article showed that pretreatment with RSV protects rat brains from ischemic damage by the SIRT1-UP2 pathway, in which RSV induces the activation of SIRT1 and decreases UP2 (106).

### *D. RSV and the cardiovascular system*

The "French paradox" refers to the observation that a lower rate of heart disease and cancer is found in Mediterranean nations, despite a diet high in saturated fat and cholesterol or high blood cholesterol levels or both (339). In these cases, RSV appears to be an excellent cardioprotective/preconditioning agent through a multitude of mechanisms. The initial characterization of RSV was due to its cardioprotective ability to act as an antiplatelet, disrupt prostanoid synthesis, and regulate lipoprotein metabolism (378). One mechanism by which RSV exerts a positive effect on the vascular system is through decreasing the expression, production, and secretion of the vasosuppressor ET-1 in endothelial cells (76, 344). Other mechanisms already detailed in this review show that RSV has a vasodilator effect by modulating the endothelium-dependent release of NO, the ER receptor, activation of a MAPK mechanism, guanylyl cyclase, and influencing intracellular $Ca^{2+}$ and $K^+$, as it is feasible that RSV could interact with a membrane receptor or ion channels. Many of the neurologic and cardiovascular preconditioning properties of RSV are based on these abilities. Preconditioning refers to the phenomenon in which an organ, such as the heart or the brain, is challenged with a brief injury exposure, such as ischemia, rendering the tissue resistant to further damage. Three phases of ischemic preconditioning have been distinguished: the initial trigger, the activation of signal transduction or biochemical changes or both (the mediators), and the action of the end effectors. Thus, the molecular underlying mechanisms are diverse and range from the influence of NO, energy metabolism, and ROS from the mitochondria to oxidative-related proteins such as Trx and protein kinase activity, such as that of PKC. In this context, the antioxidant ability of RSV (inhibition of NOX reactivity, ROS formation, ERK1/2 phosphorylation, and AP-1 activity) dampens the oxidative stress produced during cardiovascular damage and ischemia. The RSV modulation of the NO system and its ability to increase the levels of cGMP in intact vascular tissue by influencing the endothelium-derived relaxing factor–NO–GMP system reinforces its positive effects on the cardiovascular system (128). However, the property of RSV as a vasodilator is still poorly defined.

Angiogenesis refers to the natural process by which new blood vessels are formed. This process is regulated by a multifaceted mechanism involving growth factors and inhibitory peptides (72). Failure to keep angiogenesis in check results in disease due to the excessive or insufficient growth of new blood vessels as seen in cancer and cardiovascular disorders (152, 285). RSV can suppress angiogenesis, tumor growth, and wound healing (61) and is able to prevent cytokine-induced vascular leakage by downregulating the expression of adhesion molecules in TNF-α–stimulated endothelial cells (43). TNF-α–induced vascular permeability changes have been shown to be inhibited *in vitro* and *in vivo* by RSV (136). RSV has antiatherogenic effect on vascular smooth muscle cells (VSMCs), which appears to correlate with its ability to downregulate MMP9 by inhibiting AP-1 and NF-κB, decreasing ERK1/2, and inducing a cyclin-dependent $G_1$ arrest and upregulating p21 (238). RSV also functions as an antifibrotic agent in the myocardium by decreasing cardiac fibroblastic proliferation and differentiation, as shown by the ability of RSV (5–25 $\mu M$) to inhibit basal- and ANG-II–induced ERK activation (303). RSV (1–100 $\mu M$) has been shown to alter ANG-II–induced cell proliferation and ET-1 gene expression by attenuating ROS generation and subsequent ERK activation (76). RSV has also been widely reported to inhibit $Ca^{2+}$ fluxes, thereby conferring protection against ischemia (65, 81, 449). Cardiac hypertrophy is also mediated by oxidative stress–mediated signaling pathways (6, 402), and a new analogue of RSV, hapontigenin (ISO), blocks cardiac hypertrophy induced by oxidative stress (248). Similarly, ath-

erosclerosis is attributed to elevated ROS production (146). Overproduction of peroxynitrite is inhibited by RSV (1–50 $\mu M$) *via* upregulation of the intracellular GSH content (62). Rat aortic VSMCs exposed to ANG-II and EGF in the presence of RSV showed an inhibition of AKT activation due to activation of SHP2 protein-tyrosine phosphatase (SH-PTP3, SH-PTP2, PTP2C, PTP1D, and Syp), thus preventing an interaction between Gab1 and PI3K, which is necessary for further signal transduction (155). Das *et al.* (98) investigated RSV properties with relation to preconditioning of the heart. By investigating the effects of differing doses of RSV *in vivo*, they showed that RSV (2.5–5 mg/kg in rats fed over a 14-day period) regulated the protein and mRNA of redox-related proteins, such as Trx-1/2, Grx1/2, Ref-1, NF-$\kappa$B, AKT, and Bcl-2. However, in animals that were fed doses of RSV >25 mg/kg, the opposite result was seen, leading to increased death signals in the heart (114). This finding correlated with increased infarct size in the ischemia-insulted heart when exposed to 50 mg/kg of RSV. A similar study by Das *et al.* (244) also highlighted the preconditioning effect of RSV through a TRX "redox switch". The authors suggested that the switch between RSV's being an antioxidant at low concentration *versus* a prooxidant at high concentrations could be due to its ability to regulate metal redox chemistry.

### *E. RSV and inflammation*

Classically, the immune system has been divided into the innate/humoral system (phagocytoses, neutrophils, monocytes, macrophages, and natural killer (NK) cells) and the adaptive/acquired (B and T cells) system. Both immune surveillance and immunomodulation are required to monitor pathogenic states as well as to police the "self." In this respect, abnormal immune functions can be viewed as a causative effect of disease development. There can be (a) a loss of one or both processes, such as in the case of tumors; (b) an overactive response, as in arthritis; or (c) a loss of recognition, as in autoimmunity.

Cytokines are small, secreted proteins produced *de novo* in response to an immune stimulus and are often produced in a cascade by virtue of their ability to function in synergistic, antagonistic, paracrine, or autocrine fashion. Cytokines are made by many cell populations, but predominantly by helper T cells (Th) and macrophages. The combination of cytokines present determines whether the outcome of the immune response is Th1 or Th2 related. It should also be noted that new data have shown that Th17 and Treg responses are as prominent as those of Th1 and Th2. A recent report indicated that IL-17–producing T cells are heavily involved in organ-specific autoimmune diseases, a function previously attributed exclusively to IFN-secreting Th1 cells. The downregulation of the inflammatory response is mediated by (a) the inhibition of the synthesis and release of proinflammatory mediators, (b) the modification of eicosanoid synthesis, (c) the inhibition of activated immune cells, and (d) the inhibition of systems, such as iNOS and COX-2, *via* repression of redox-regulated transcription of genes, such as *NF-κB* and *AP-1*. NF-$\kappa$B controls the expression of genes that encode the proinflammatory cytokines (*e.g.*, IL-1, IL-2, IL-6, IL-17, and TNF-$\alpha$), chemokines (*e.g.*, IL-8, MIP-1$\alpha$, MCP1, RANTES, and eotaxin), adhesion molecules (*e.g.*, ICAM, VCAM, and E-selectin), inducible enzymes (*e.g.*, COX-2 and iNOS), growth factors, and some of the acute-phase proteins and immune receptors, all of which play critical roles in controlling the inflammatory processes.

Although most of the research to date with regard to understanding the effects of RSV on the immune system has included only experiments performed in Petri dishes, some animal models have suggested that RSV can have a potent effect. In pancreatic cells, RSV upregulates macrophage inhibitory cytokine (MIC-1) and appears to be a key player in the growth and inhibition of these cells (149). RSV can also modulate IL-17, as shown in three studies. With an animal model of multiple sclerosis, RSV was shown to decrease the symptoms, and RSV induces caspase Fas-apoptosis in activated T cells and, to a lesser extent, in inactivated ones through the activation of AhR and ER. TNF-$\alpha$; IFN-$\gamma$, IL-2, -9, -12, and -17; MIP-1$\alpha$; monocyte chemoattractant protein-1 (MCP-1); RANTES; and eotaxin were modulated by RSV (189). Another study, also using an animal model of multiple sclerosis, concluded that RSV offered protection by increasing the IL-17$^+$/IL-10$^+$ T cells and CD4$^-$IFN-$\gamma^+$ while repressing macrophage IL-6 and IL-12/23 p40 expression; others have shown that RSV inhibits high glucose–induced PI3K/AKT/ERK-dependent IL-17 expression in primary mouse cardiac fibroblasts (373, 421).

Chemokines are chemotactic cytokines that direct migration of leukocytes, activate inflammatory responses, and participate in the regulation of tumor growth. Chemokines exert their migration-inducing properties on leukocytes through binding to chemokine receptors. IL-8/CXCL8 was the first chemokine identified as being able to stimulate endothelial cell chemotaxis, proliferation, and angiogenesis *in vivo*. RSV suppressed *IL-8* gene transcription in TPA-treated human monocytic cells (368). This suppression is partially due to the inhibition of AP-1. In addition, the chemokine fractalkine is regulated by RSV on TNF-$\alpha$ stimulation in human umbilical vein endothelial cells and in THP-1 (human monocytic) cells (284). Fractalkine activates platelets and induces leukocyte adhesion to the endothelium, and expression of fractalkine and its receptor, CX3CR1, is elevated in coronary artery disease. Fractalkine also induces vascular dysfunction by stimulating vascular ROS, resulting in reduced NO bioavailability. RSV strongly suppresses TNF-$\alpha$–induced fractalkine expression in endothelial cells by suppressing NF-$\kappa$B and Sp-1, thereby abrogating the number of fractalkine-positive endothelial cells, CX3CR1-positive cells, and monocytes adhered to human umbilical vein endothelial cells. Additionally, the monocyte chemotactic protein-1 and its receptor, CCR2, play a key role in atherosclerosis (90). RSV inhibits the monocyte CCR2 binding activity in a manner dependent on NO, MAPK, and PI3K. In contrast, RSV inhibits CCR2 mRNA in a manner independent of NO and MAPK but dependent on PI3K. These inhibitory effects of RSV on chemokines-receptor binding and expression may partially contribute to its cardiovascular protective activity *in vivo*. With C5 anaphylatoxin (C5a)-stimulated primary neutrophils and a mouse model of acute peritonitis, two new targets of RSV were identified; it can inhibit sphingosine kinase (SphK) membrane localization, activity ($IC_{50}$, $\sim 20\,\mu M$), and SphK1-mediated $Ca^{2+}$ release through the inhibition of phospholipase D activity and ERK1/2 phosphorylation (193). Furthermore, RSV attenuated the release of IL-1$\beta$, TNF-$\alpha$, IL-6, and MIP-1$\alpha$, suggesting that the antiinflammatory actions

of RSV are, in part, mediated by modulation of SphK and phospholipid D and can regulate neutrophil activity.

Toll-like receptors (TLRs) are type I transmembrane proteins that function in the adaptive and innate immune response. They form homo- or heterodimers, and each dimer has a particular ligand specificity and will recruit the cytoplasmic adapter molecules MyD88, TIRAP (Mal), TRIF, and TRAM. The adapters activate downstream amplification signal networks, such as IRAK1, IRAK4, TBK1, and IKKi, leading to regulation of gene expression. RSV influences the MyD88 and Toll/IL-1R domain-containing adaptors that induce the IFN-$\beta$ (TRIF) pathways, leading to the expression of *proinflammatory cytokines and type I IFN* genes, respectively (446). LPS can act through TLR4 and induce IL-6 expression *via* ERK1/2, p38 MAPK, NF-$\kappa$B, and C/EBP in VSMCs. The ability of LPS to activate these pathways can be reversed by RSV (379). Furthermore, on RSV treatment, the phagocytic activity of macrophages against bacteria is reduced (194). Radkar *et al*. (335) reported RSV-induced cell death in TLR4-null macrophages, which could be prevented by the addition of TLR4. These results raise the possibility that certain dietary phytochemicals can modulate TLR-derived signaling and inflammatory target-gene expression and can alter the susceptibility to microbial infection and chronic inflammatory diseases.

### F. The antimicrobial activity of RSV

As RSV is a natural defense molecule against pathogens in plants, it is rational to assume that it will have antipathogenic properties in all macrobiotic systems. RSV is currently in phase II clinical trials for treating herpes simplex virus (a nuclear replicating, icosahedral, enveloped DNA virus). The herpes simplex virus, HSV-1, is transmitted through saliva, and HSV-2 is transmitted through sexual contact, causing widespread infection throughout the population. The use of RSV in treating HSV has been based on findings that RSV can abrogate NF-$\kappa$B activation in HSV-infected cells. As HSV-1 replication uses the I$\kappa$B kinase-I$\kappa$B-RelA/p65 pathway, this is an important finding and explains how RSV can inhibit reactivation of the latent virus (109, 123). It has also been shown that RSV is effective against human herpesvirus replication in a dose-dependent and reversible manner, when given during the first 30 h, and this appears to abrogate IE62 synthesis (110). The severe acute respiratory syndrome (SARS) virus was also shown to be negatively regulated by RSV in the Vero E6 cell model of infection (250). Human papillomavirus-16 (HPV-16) oncoproteins contribute to enhanced angiogenesis in cervical cancer cells *via* HIF-1$\alpha$–dependent VEGF expression. RSV suppresses HPV-16 E6- and E7-induced HIF-1$\alpha$–mediated angiogenic activities and is a potential chemotherapeutic agent for human cervical cancer (401). RSV can also be effective against human herpesvirus 6 (HHV-6)-infected lymphoblasts (288). A recent study using an analogue of RSV showed that its effectiveness against influenza A virus in A549 cells *via* the AKT-STAT1 pathway lies in its ability to decrease the production of CCL5/RANTES (183). Furthermore, RSV has been shown to be a potent, synergistic inhibitor of human immunodeficiency virus (HIV)-1 infection when given in combination with nucleoside analogues (172, 196). RSV can also target cellular genes expressed in latently infected cells (225). In light of new reports of the structure of HIV, it would be worthwhile to evaluate such data with a view toward future translational developments (453).

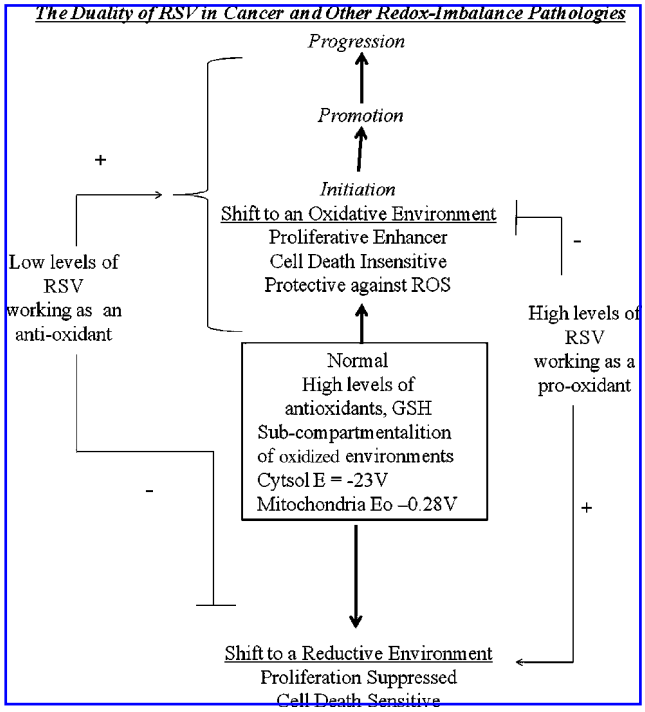



**FIG. 10. A slight prooxidant intracellular milieu is invariably associated with cancer cells and has been linked to a slight increase in the ratio of superoxide to hydrogen peroxide, the two main ROS.** This mild oxidative stress results in DNA damage and promotes proliferation, while inhibiting apoptotic execution. A tilt in the ratio toward hydrogen peroxide results in increased reductive stress and facilitates apoptotic signaling. The involvement of antioxidant defenses is highlighted in this context.

Another drug target is ribonucleic acid (RNA), which is a potential target for treating yeast and other fungal infections. Of specific interest are the group I intron RNAs, which are a unique class of RNA molecules that undergo self-catalytic activity because of a unique folded structure that catalyzes a number of cellular reactions. These RNA molecules have been found in human pathogens like *Candida albicans*, but not in human cells, suggesting that they could be alternative therapeutic targets (417). With the densitometry method, the binding efficacy of RSV to RNA was found to be high, indicating that RSV may bind group I intron RNAs and work as an antiyeast treatment. RSV is directly cytotoxic to fungi and can alter fungal morphogenesis. Intriguingly, compounds based on RSV, such as $\varepsilon$-viniferin and $\alpha$-viniferin and *trans*-pterostilbene, are more potent antifungal agents than RSV, suggesting that RSV analogues should be examined as potential candidates. In the future, the ability of RSV to induce autophagy may also be important in immune recognition by regulating TLR activity, which is particularly intriguing in light of a recent report showing that components of single-stranded RNA (ssRNA) viruses in endosomes can be detected by TLRs (239). This recognition requires that TLR7 triggers viral replication intermediates to be autophagocytosed. This, in turn, triggers the production of IFN-$\gamma$. It would therefore be

of great interest to evaluate the potential of RSV in modulating or enhancing such events.

### G. The anticancer properties of RSV

In current theories explaining the genesis of cancer tumor biology, the principal models are "stem cell theory" and "dedifferentiation." The central dogma regarding cancer establishment and perpetuation is that it occurs *via* the stages of "initiation, promotion, and progression," and that targeting one, if not all, of these steps would be advantageous in fighting cancer. Tumor promotion is believed to be an essential process in multistage cancer development, enabling clonal expansion and genetic instability of preneoplastic and premalignant cells. Whether this expansion comes from one cell or a few cells has been a matter of debate over the past decade, and a more-cautious outlook has now been presented: cells found in a cancer mass can be either homogeneous or heterogeneous in their abnormalities. Aneuploidy has an antitumorigenic function in chemical or genetic cancers [*e.g.*, loss of p16/ARF (ADP ribosylation factors]. In contrast, in tumors that are spontaneous and age related, aneuploidy has a promoting effect (192, 431). Another theory that has been growing in acceptance is that of "cooperation," which combines the biologic principal that tumors are heterotypic and the view that cancer works on a darwinian principle (105). It has been predicted, at least mathematically, that neighboring cells protect one another from the immune response and that both cells are needed, as neither are capable of independent survival. Interestingly, it has been reported that the presentation of an "angiogenic switch" (an imbalance between pro- and antiangiogenic factors) triggers invasive tumor growth (190, 426). What is also becoming increasingly clear is that ROS are involved in the progression of cancer. In this context, the Warburg theory, which proposes that cancers have a high metabolic rate and that mitochondria are more hyperpolarized in cancer, as compared with normal cells, has been revisited (141). The proposal that metabolic oxidation/reduction reactions involving ROS could contribute to carcinogenesis and progression to malignancy has gained scientific acceptance, and it is now believed that ROS could act as both initiator and promoter of carcinogenesis (Fig. 5). In this regard, low concentrations of RSV have been shown to inhibit cell death by inhibiting ROS generation in cancer cells, suggesting a word of caution for its potential use as an anticancer agent (Fig. 10) (8, 9). This is further exemplified by the findings of Wang *et al.* (430) that, in the LNCaP cell line, RSV at $>25\,\mu M$ can induce cell death, with an inhibitory effect seen after 96 h with $5\,\mu M$ (430). However, different results were obtained when RSV was administered in xenografted mice (3–6 mg/day; plasma level of $1.3 \pm 0.336\,\mu M$). Although an initial decrease in growth was observed, a decrease in apoptosis and an increase in angiogenesis were seen over time. This study further justifies concern about the use of RSV in the management of cancer.

*In vivo* data show that RSV can block the three stages of carcinogenesis: initiation, promotion, and progression. Although this substantiates its claim as a chemotherapeutic agent, its prophylactic activity remains ill defined (197). RSV has the unique ability of targeting a number of cancers *via* the multitude of pathways already detailed in this review. RSV has also been shown to override the multidrug resistance–associated proteins (54, 436). Furthermore, many solid tumors contain at least a hypoxic center. The expression of hypoxia-inducible factor-1alpha (HIF-1$\alpha$) correlates with an aggressive tumor phenotype and an increase in VEGF expression (102, 326). HIF-1$\alpha$ regulates transcription in response to oxygen levels *via* the PHD-VHL/Elongin-C/Elongin-B E3 ubiquitin ligase and the proteasome. Recently, Semenza *et al.* (259) illustrated that inhibition of heat-shock protein 90 leads to an independent degradation of HIF-1$\alpha$ *via* the receptor of activated protein kinase C-1 (RACK-1). Park *et al.* (186) reported that HIF-1$\alpha$ participates in the cellular processes of the hypoxia-induced resistance to RSV in Hep3B (hepatoma), Caki-1 (renal carcinoma), SK-N-MC (neuroblastoma), and HEK293 (human embryonic kidney) cell lines (186). In Lovo cells (colon carcinoma cells), however, it was shown that RSV can decrease migration, adhesion, and invasion by decreasing the expression of VEGF, MMP9, and MMP2, under hypoxic conditions or on treatment with 2,2′-dipyridyl treatment (an iron chelator) (437). RSV has also been shown to induce the expression of the nonsteroidal antiinflammatory drug–activated gene-1 (NAG-1), a member of the TGF-$\beta$ superfamily, *via* p53 activity, thereby inducing cell death and acting as an antitumorigenic agent (32). Others have shown similar findings by using the invasive inducer lysophosphatidic acid (LPA), which increases HIF-1$\alpha$ levels under hypoxia, resulting in an increased expression of VEGF protein and mRNA (311). In this study, RSV inactivated p42/p44 MAPK and p70S6K and enhanced the degradation of HIF-1$\alpha$, thereby decreasing VEGF expression and cell migration. RSV is also able to affect HIF-1$\alpha$ in human tongue squamous cell carcinomas and hepatoma cells by inhibiting the basal level and hypoxia-induced HIF-1$\alpha$ protein accumulation in cancer cells. RSV accomplishes this by decreasing the half-life of HIF-1$\alpha$ through enhancing protein degradation by the 26S proteasome system (451). In this system, RSV inhibits the hypoxia-mediated activation of ERK1/2 and AKT, thereby decreasing HIF-1$\alpha$ protein accumulation and VEGF transcriptional activation and resulting in a decrease in the invasiveness of cancer cells.

RSV can induce death *via* cell-cycle deregulation or depletion of prosurvival proteins through either p53-dependent or -independent mechanisms. The latter was illustrated by the work of Mahyar-Roemer *et al.* (265) showing that in HCT116 (colon) cells, RSV ($100\,\mu M$) induced an initial proliferation and differentiation, leading to a p53-independent apoptosis. This induction involved Bax activation. Thus, the application of RSV as an anticancer agent has been proposed, based on its efficacy on a range of genetic phenotypes. It also appears to be an efficient cell-death sensitizer, as demonstrated when RSV was given together with classic drug therapies, such as 5-fluorouracil (5-FU), which sensitized a number of cell lines to these drugs (438). More recently, RSV has been shown to overcome imatinib resistance in CML (330) and to enhance apoptosis induced by 17-allylamino-17-demethoxygeldanamycin (17-AAG) in CML cells by down-regulating Hsp70 (73).

Another form of cancer treatment is radiotherapy (internal and external), which is especially useful in cancers that are hard to reach by surgery or are unresponsive to chemotherapeutics or in cases in which the drugs are unable to cross the blood–brain barrier. However, its use is limited because of its severe toxicity toward normal cells at high dosages and its minimal effects at low dosages. The properties of

radiosensitization are enhanced with RSV, and Baatout *et al.* (29) illustrated that RSV can sensitize HeLa, K-562, and IM-9 cells to a dosage of 0–8 Gy of x-rays (29). Apparently, RSV can also enhance the cytotoxic response of ionizing radiation in cell lines. In addition, DU145 cells (resistant to ionizing radiation–induced cell death) pretreated with RSV undergo significantly enhanced cell death, as well as synergistically inhibited cell survival (355). RSV also potentiates ionizing radiation–induced ceramide accumulation by promoting its *de novo* biosynthesis. This confirms that ceramide is an effective mediator of the anticancer effects of RSV, as suggested by many other publications (347). Aziz *et al.* (27) showed that in response to UVB irradiation, survivin could be downregulated by pretreatment with RSV and that the downregulation of SMAC/DIABLO was reversed and restored by RSV. Afaq *et al.* (2) demonstrated that topical applications of RSV (25 $\mu$mol/0.2 ml acetone/mouse) on SKH-1 hairless mouse inhibited damage from short-term exposure to UVB (180 mJ/cm$^2$). Reagan-Shaw *et al.* (337) showed that long-term exposure to UVB was also abrogated by topical application of RSV and that this abrogation appeared to be mediated by the cyclin cell-cycle network and MAPK pathway. Another method of treatment was explored, in which RSV was used to induce the expression of TNF-$\alpha$ from the radio- and chemoinducible cancer gene–therapy vector Ad.Egr. TNF (50).

In cancer drug development, the modification of promising lead compounds to reduce toxicity and increase efficacy has a self-driving momentum. RSV has also been modified, and several compounds have been evaluated. Vaticanol C (Vat-C) is a novel RSV tetramer (370). With a p53-mutated metastatic mammary cancer mouse model, Vat-C was shown to induce apoptosis in the tumors, although the tumor volumes did not change, and the metastatic potential of the cancer decreased. Notably, the microvessel density in tumors and the lymphatic vessels having intraluminal tumor cells were reduced in the treated group. One indicator of the aggressiveness of a tumor is whether it still expresses hormone receptors or has become independent. Without endogenous hormones and according to cellular specificity, the superagonistic activity of RSV may act in an opposing fashion to prevent tissue senescence and apoptosis. However, when stress signals overcome proliferative signals or when an absence of hormones is noted, the RSV-induced pathway may switch to an apoptotic one. The ability of RSV to potentiate the effectiveness of apoptotic or cytostatic drugs requires pre- or cotreatment, and the addition of RSV after the stress stimulus is ineffective. Such observations indicate that RSV induces and potentiates the event cascades directed toward clusters of gene transcription–mediated events. The actions of RSV are cell specific, as it is toxic to tongue squamous carcinoma, more so than to gingival fibroblasts (30), and to leukemia cells, but not to normal hematopoietic progenitors or peripheral blood lymphocytes (51, 87). This is an important nuance in the treatment of malignancies. A number of cancer cells that form dietary-restriction–resistant tumors carry mutations resulting in the constitutive activation of the PI3K pathway and proliferate in the absence of insulin or IGF-1. Dietary restriction has been shown to have no effect in the prostate PTEN-null mouse model, but could decrease the tumor burden in a lung cancer lacking constitutive PI3K signaling. The authors also showed that expression of an activated mutant allele of PI3K with wild-type PI3K or the restoration of PTEN expression in a PTEN-null cancer cell line restored dietary-restriction sensitivity. It would also be of interest to study these findings in other models known to contain AKT mutations, such as the rare activating mutation of AKT1 (E17K) reported in breast, ovarian, and colorectal cancers, melanoma, and lung cancer, and in models with serine/threonine kinase AKT2-BRCA1 deficiency, which activates the AKT oncogenic pathway. This could help explain some of the conflicting results seen when using RSV as an anticancer agent. To date, one study has tested 178 human cancer cell lines, including the entire NCI60 cell line collection and found the AKT1 (E17K) in WM46 and the D40 melanoma cell line (100).

Furthermore, RSV has been reported to be a potential agent in the treatment of cachexia (405, 440). RSV can inhibit proteolysis-inducing factor (PIF), ANG-II, and TPA-induced NF-$\kappa$B activation in C2C12 myotubes. RSV (1 mg/kg per day) was reported to reduce the weight loss seen in MAC 16 tumor-bearing rats, which are known to respond to RSV, although in Yoshida AH-130 ascites hepatoma or Lewis lung carcinoma models RSV at 1–25 mg/kg per day, it has no effect (69). These results suggest that RSV may positively influence the secondary effects associated with cancer and warrant further investigation, especially in light of the ability of RSV to regulate glucose metabolism *via* sirtuins. Overall, this suggests that RSV has the rare property of targeting hormone and nonhormone, p53-dependent and -independent expressing human tumors.

## V. Concluding Remarks and Implications

As seen throughout this review, the ability of RSV to affect numerous pathways and even aid in the elucidation of new ones underpins its potential as a therapeutic agent. However, these effects are dependent on the dose and the model. To summarize, the principal enzyme targets inhibited by RSV are the following: COX and LOX (3.7 and 15 $\mu M$), PKCs and p56lck (40 and 60 $\mu M$), ERK1 (37 $\mu M$), JNK1 (50 $\mu M$), p38 (50 $\mu M$) IKKB (1 $\mu M$), Src (20 $\mu M$), Stat3 (20 $\mu M$), ribonucleotide reductase (50 $\mu M$), DNA polymerases (3.3 and 5 $\mu M$), PKD (35–50 $\mu M$), PKC$\alpha$ ($<$10 $\mu M$), NQOR (35–50 n$M$), aromatase (25 $\mu M$), NF-$\kappa$B (100 n$M$/1 $\mu M$), whereas RSV activates Sirt2/SIRT1 (100 $\mu M$), adenylate cyclase (0.8 $\mu M$), and AMPK (50 $\mu M$) (324). Furthermore, the following questions have yet to be fully answered: (a) what dosage is required for RSV to be effective, (b) is it RSV or its metabolites that are effective, (c) does RSV interact with dietary or other drugs, and (d) what are the side effects of long-term use? These finer points of RSV use are well reviewed by Bertelli (42) in the context of the effect of RSV in cardiovascular disease: very low doses of RSV ($<$1.72 $\mu M$) have a positive effect on the cardiovascular system in rats and humans. This stands in stark contrast to the cytotoxic concentration of RSV, suggesting that, other than in chemotherapy, the RSV applied in its native form and at lower dosage are the most effective. Many of the effects elicited upon exposure to RSV may be mediated by the main metabolites of RSV: *trans*-RSV-3-*O*-glucuronide and *trans*-RSV-3-sulfate. While both the sulfate conjugates as well as glucoronides of RSV have been implicated in its various biological activities, the possibility of deconjugation to generate free RSV as a probable active species has also been recently raised.

It is hypothesized that RSV as a combination therapeutic will be beneficial, although clinical trials are lacking. The Food and Drug Administration (FDA) does not recognize RSV as dietary supplement, as it was not marketed before the enactment of the Dietary Supplement Health & Education Act, in 1994. RSV has instead been given investigational new-drug status so that it may be tested in clinical studies. This is a prudent move, considering the reports that RSV can work synergistically with known drugs. A recent study has shown that RSV has effects on the pharmacokinetics of diltiazem and its active metabolite, desacetyldiltiazem, in rats because of the inhibition of both the cytochrome P450 (CYP) 3A4–mediated metabolism and the efflux pump P-glycoprotein (P-gp) in the intestine or liver or both. If these results are confirmed in clinical experiments, the dosage of diltiazem should be readjusted when diltiazem is used concomitant with RSV.

The study of Pignatelli *et al.* (322), based on the central Italian population, also highlighted the point that RSV should be considered together with other dietary agents, such as caffeine, and vitamins, which may work together to prevent oxidative stress. It has also been suggested that long-term absorption of RSV could allow tissue accumulation and that RSV could act with other agents to enhance the effect of nutritional supplements. However, caution must be used, as few long-term studies have been performed on the side effects of RSV consumption. Considering that it can alter longevity gene expression, the immune system, and neuronal regeneration and has estrogen-like properties, it would be surprising if some unforeseen side effects were not obtained. Interestingly, wine drinking can have an adverse effect in light smokers while being preventive in heavy smokers, most likely due to the ability of RSV to modulate CYPs and thus the metabolism of polycyclic aromatic hydrocarbons (212). RSV has also been shown to be able to blunt acute respiratory syndrome by inhibiting leukocyte–endothelium interactions, reducing blood viscosity and edema, and the infiltration of leukocytes and ROS from cells in the lung. Furthermore, from a toxicologic standpoint the safety of RSV supplements during fertility, pregnancy, and lactation has not been established. Some mouse studies showed that RSV can suppress fertility and has an adverse affect on male and female reproductive organs in outbred CD-1 mice (233, 298). It should also be noted that other studies illustrate that RSV can increase sperm output and protect sperm from apoptosis caused by physical damage, as well as protecting the fetus from harmful environmental factors, such as smoking. As RSV affects sirtuins, it is worth noting that this family of proteins has functions in fertility as well as in the generation of offspring sensitivity to insulin, as shown by two recent studies. According to Allsopp *et al.* (89), in the germline of $Sirt^{-/-}$ mice, markedly attenuated spermatogenesis was noted, but not oogenesis. Furthermore, the proportion of mature sperm with elevated DNA damage ($\sim$7.5% of total epididymal sperm) was significantly increased in adult $Sirt1^{-/-}$ males. Viable animals were obtained from $Sirt1^{-/-}\times$wild type and $Sirt1^{-/-}\times Sirt1^{-/-}$ crosses, but when IVF was performed with $Sirt1^{-/-}$ sperm or oocytes or both, the efficiency of producing both two-cell zygotes and viable offspring was decreased. Ozanne *et al.* (273) showed that limiting protein and growth during lactation increases longevity when done postnatally (PLP), whereas *in utero* growth restriction followed by "catch-up" growth (recuperated group) decreases the lifespan. This may be related to the altered insulin response, as it was noted that PLP animals had improved insulin sensitivity and significant upregulation of insulin receptor-$\beta$, IGF-1R, AKT1, AKT2, and AKT phosphorylated at Ser473, as well as Sirt1, catalase, Cu/ZnSOD, and GPX1. In contrast, recuperated animals had increased MnSOD and increased fasting glucose concentration, whereas the insulin levels remained similar to those of the control group. It is tempting to speculate that RSV will have an effect on these systems and that the regulation of the vitagenen systems (cytoprotective Hsp70, HO-1, Trx, and sirtuins), which are emerging as a neurohormetic target for aging, will be modulated at an early time during development.

As the studies progress, potential delivery methods for RSV as well as new analogues to increase the efficacy of the parent compound are being developed. The latter is highlighted by studies investigating the topical and oral application of RSV. When RSV is given by direct oral absorption, such as in the case of 1 mg of RSV in 50-ml solution and retained in the mouth for 1 min before swallowing, 37 ng/ml of RSV was observed in the plasma. The equivalent concentration of RSV in pill form is only achieved with 250 mg of RSV (18). However, the fact that RSV is rapidly metabolized has led to the argument against the use of RSV in the clinical setting. This has driven drug development to develop different RSV formulations, such as methylated RSV, which is metabolized slower, or as the patented oral formulation of SRT-501 (3–5 g), developed by Sirtris Pharmaceuticals, Inc., which reaches 5 to 8 times higher blood levels, close to the necessary concentrations used in *in vitro* studies (119). This has also led to RSV-based analogues, some of which are in clinical trials for cancer, AD, and diabetes. The FDA has also granted Sirtris Pharmaceuticals, Inc., an orphan-drug designation for RSV in the treatment of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes syndrome, or MELAS, broadening the range of RSV use for metabolic syndrome, aging, and obesity.

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**Abbreviations Used**

5-FU = 5-Fluorouracil
8-oxo-dGTP = 8-oxo-2′-deoxyguanosine triphosphate
AA = arachidonic acid
AA-CoA = acetyltransferase, acetyl-CoA C-acetyltransferase
AD = Alzheimer's disease
AhR = aryl hydrocarbon receptor
AIF = stress-apoptosis–inducing factor
ANG-II = angiotensin II
AP-1 = activator protein-1
AP-2 = activator protein-2
Ape1/Ref-1 = purinic endonuclease 1/redox factor-1
ARE = antioxidant responsive element
ARNT = AhR nuclear translocator
ASK1 = apoptosis signal–regulating kinase 1
ATF-associated TPA response element = TRE (5′-TGAG/CTCA-3)
ATM = ataxia telangiectasia mutated
ATP = adenosine triphosphate
ATR = ataxia telangiectasia
BER = base excision repair
BRCA1 = breast cancer type 1 early onset
BRCA2 = breast cancer type 2 susceptibility protein
C/EBP = CCAAT/enhancer-binding protein
$Ca^{2+}$ = calcium
$Ca^{2+}$/CaM/CaMKIα = $Ca^{2+}$/calmodulin-dependent protein kinase II
CaM kinase = calcium-activated kinase
cAMP = cyclic adenosine monophosphate
Cdk = cyclin-dependent kinase
c-FLIP = FLICE-inhibitory protein
cGMP = cyclic guanosine monophosphate
ChK1 = checkpoint kinase 1
ChK2 = checkpoint kinase 2
CHOP/GADD153 = small nuclear protein CCAAT/enhancer-binding homologous protein
cIAP-2 = apoptosis protein 1
COX-2 = cyclooxygenase-2
cPLA2 = phospholipase A2
CREB = cAMP response element–binding
CRTC2/TORC2 = CREB-regulated transcription co-activator 2
Cu(II) = copper II
CYP = cytochrome P450 monooxygenase
DAG = diacylglycerol
DES = diethylstilbestrol
DISC = death-inducing signaling complex
DMBA = 7,12-dimethylbenz[a]anthracene
DNA-PK = DNA-dependent protein kinase
DR4, 5 = death receptor 4, 5
DSBs = DNA double-strand breaks
E2 = 17β-estradiol
E2F = E2F family of DNA-binding transcription factors
EGF = epidermal growth factor
EGFR = EGF receptors
Egr-1 = early growth-response gene
EpRE = electrophile responsive element
ER = estrogen receptor
ET-1 = endothelin-1
FADD = fas-associated protein with death domain
cyt.c = cytochrome *c*
FADD-DN = fas-associated death domain-dominant negative
Fe-S = iron-sulfur
FOXO = forkhead box O-class
GADD45 = growth arrest and DNA damage gene 45
GLUT-4 = glucose transporter-4
GSSG = glutathione disulfide
GSSH = glutathione persulfide
GSH = glutathione
GMP = guanosine monophosphate
GPX = glutathione peroxidases
GTP = guanosine-5′-triphosphate
$H_2O_2$ = hydrogen peroxide
HATs = histone acetyltransferases
HD = Huntington's disease
HDAC = $NAD^+$-dependent histone deacetylases
HIF-1α = hypoxia-inducible factor 1-α
H2AX = histone 2AX
HO-1 = heme oxygenase-1
HR = homologous recombination
Hsp27 = heat-shock protein 27
HSV = herpes simplex virus
I(KATP) = anoxia-induced KATP-channel current
IAPs = inhibitors of apoptosis
IARC = International Agency for Research on Cancer
ICAM = intercellular adhesion molecule 1
IFNα = interferon alpha
IGFBP-1 = insulin-like growth factor–binding protein
IGF-I = insulin-like growth factor-I
IKK = IκB kinase complex
IL-1β = interleukin-1β
IL-2 = interleukin-2
IL-6 = Interleukin-6
IL-8 = interleukin-8
iNOS = inducible nitric oxide synthase
IP3 = inositol-triphosphate 3
JDP = Jun dimerization partner
JNK = c-Jun NH(2)-terminal kinase
KATP = ATP-sensitive potassium
L-NAME = *N*-nitro-L-arginine methyl ester
LOX = lipoxygenase
LPS = lipopolysaccharide
Maf = avian musculoaponeurotic fibrosarcoma protooncogene
MAPKs = mitogen-activated protein kinases
MCP1 = monocyte chemotactic protein-1α
MIC-1 = macrophage inhibitory cytokine
MIP-1α = macrophage inflammatory protein-1α
MMP-9 = matrix metalloprotease-9
MnSOD/SOD2 = mitochondria SOD
MPP+ = 1-methyl-4-phenylpyridinium
MPT = mitochondrial permeability transition pore

**Abbreviations Used** (Cont.)

MSK-1 = mitochondrial lysine-tRNA synthetase
MTH1 = MutT homologue 1
NHE-1 = $Na^+/H^+$exchanger-1
$NAD^+$ = nicotinamide adenine dinucleotide
NADPH = nicotinamide adenine dinucleotide phosphate
NAG-1 = nonsteroidal antiinflammatory drug–activated gene-1
NF-$\kappa$B = nuclear factor-kappa B
NHEJ = nonhomologous end joining
Nbs1 = Nijmegen breakage syndrome
NK = natural killer
NO = nitric oxide/nitrogen monoxide
NOX = NAD(P)H oxidase
NQO1 = quinone reductase 1
Nrf-2 = NF-E2–related factor 2
NSAID = nonsteroidal antiinflammatory drug
$O_2^-$ = superoxide
$O_2$ = oxygen
ODC = ornithine decarboxylase
OH = hydroxyl
$ONOO^-$ = peroxynitrite
oxLDL = oxidized low-density lipoproteins
p21 = p21waf1/cip1
p70S6K = p70 S6 kinase
PAR = branched polymer of repeating ADP-ribose units
PARG = poly (ADP-ribose) glycohydrolase
PARP-1 = poly-ADP-ribose polymerase-1
PC = phosphatidylcholine
PD = Parkinson's disease
PDGF = platelet-derived growth factor
PE = phosphatidylethanolamine
PELP1 = proline-, glutamic acid-, and leucine-rich protein-1
PEPCK = phosphoenolpyruvate carboxykinase
PGC-1$\alpha$ = PPAR$\gamma$ coactivator-1$\alpha$
PGE2 = prostaglandin E2
PGF2$\alpha$ = prostaglandin F2$\alpha$
PGG2 = prostaglandin G2
PGH2 = prostaglandin H2
PH = pleckstrin homology
phorbol ester = 12-*O*-tetradecanoyl phorbol-13-acetate/TPA
PI3K = phosphoinositide 3-kinase
PIP2 = phosphatidylinositol 4,5-bisphosphate
PKA = protein kinase A
PKC = protein kinase C
PKC$\alpha$ = protein kinase C-$\alpha$
PLC = phospholipase C
PMA = phorbol myristate acetate
$pO_2$ = partial pressure of oxygen
PPAR$\gamma$ = peroxisome proliferator–activated receptor-$\gamma$
PKD = protein kinase D
PS = phosphatidylserine
pS6RP = phospho-S6 ribosomal protein
PSA = prostate specific antigen
PTEN = phosphatase and tensin homologue
PTP1B = protein tyrosine phosphatase-1B
PXR = pregnane X receptor
RACK-1 = receptor of activated protein kinase C-1
RANTES = regulated on activation, normal T-cell expressed and secreted
Raptor = rapamycin sensitive *via* FK506-binding protein
Rb = retinoblastoma
Rector = rapamycin insensitive
RNS = reactive nitrogen species
ROS = reactive oxygen species
RSS = sulfur-reactive species based
RSV = resveratrol
SARS = severe acute respiratory syndrome
SERM = selective estrogen-receptor modulator
SHP2/SH-PTP3/SH-PTP2/PTP2C/PTP1D = tyrosine phosphatase
STAT = signal transducers and activators of transcription
SIRT = sirtuin
Skp2/p45 = S-phase kinase-associated protein 2
SOD = superoxide dismutase
STZ = streptozotocin induced
SUR = sulfonylurea receptor-1
Syp = synaptophysin
T4 = tetraiodothyronine 5′ deiodinases
TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TGF-$\alpha$ = transforming growth factor-$\alpha$
Th = T-helper cells
TLR = Toll-like receptor
TNF-$\alpha$ = tumor necrosis factor, cachexin or cachectin
TPA = tissue plasminogen activator
TRAMP = transgenic adenocarcinoma mouse prostate male
TRIF = TIR-domain–containing adapter-inducing interferon-$\beta$
Trx = thioredoxin
TrX-1 = thioredoxin-1
TrxR = thioredoxin reductase
Txnip = thioredoxin-interacting protein
UCP-1 = uncoupling protein-1
VSMC = vascular smooth muscle cell
Vat-C = vaticanol C
VCAM-1 = vascular endothelial adhesion molecule-1
VEGF = vascular endothelial growth factor

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