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Angiotensin II blockade: how its molecular targets may signal to mitochondria and slow aging. Coincidences with calorie restriction and mTOR inhibition

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de Cavanagh EM, Inserra F, Ferder L. Angiotensin II blockade: how its molecular targets may signal to mitochondria and slow aging. Coincidences with calorie restriction and mTOR inhibition. Am J Physiol Heart Circ Physiol 309: H15–H44, 2015. First published May 1, 2015; doi:10.1152/ajpheart.00459.2014.—Caloric restriction (CR), renin angiotensin system blockade (RAS-bl), and rapamycin-mediated mechanistic target of rapamycin (mTOR) inhibition increase survival and retard aging across species. Previously, we have summarized CR and RAS-bl’s converging effects, and the mitochondrial function changes associated with their physiological benefits. mTOR inhibition and enhanced sirtuin and KLOTHO signaling contribute to the benefits of CR in aging. mTORC1/mTORC2 complexes contribute to cell growth and metabolic regulation. Prolonged mTORC1 activation may lead to age-related disease progression; thus, rapamycin-mediated mTOR inhibition and CR may extend lifespan and retard aging through mTORC1 interference. Sirtuins by deacetylating histone and transcription-related proteins modulate signaling and survival pathways and mitochondrial functioning. CR regulates several mammalian sirtuins favoring their role in aging regulation. KLOTHO/fibroblast growth factor 23 (FGF23) contribute to control Ca2+, phosphate, and vitamin D metabolism, and their dysregulation may participate in age-related disease. Here we review how mTOR inhibition extends lifespan, how KLOTHO functions as an aging suppressor, how sirtuins mediate longevity, how vitamin D loss may contribute to age-related disease, and how they relate to mitochondrial function. Also, we discuss how RAS-bl downregulates mTOR and upregulates KLOTHO, sirtuin, and vitamin D receptor expression, suggesting that at least some of RAS-bl benefits in aging are mediated through the modulation of mTOR, KLOTHO, and sirtuin expression and vitamin D signaling, paralleling CR actions in age retardation. Concluding, the available evidence endorses the idea that RAS-bl is among the interventions that may turn out to provide relief to the spreading issue of age-associated chronic disease.

mechanistic target of rapamycin; vitamin D; caloric restriction; renin-angiotensin system

EFFORTS AIMED AT DECODING the mechanisms that underlie the aging process have unveiled three interventions that can increase survival and retard age-related diseases from lower organisms to mammals, i.e., caloric restriction (CR) (84, 85, 140, 160, 334), mechanistic target of rapamycin (mTOR; originally mammalian TOR) inhibition by rapamycin (173, 196, 281), and renin-angiotensin system blockade (RAS-bl) (20, 21, 26, 63, 253, 284, 354), although the latter has been less studied. In light of these findings, some intriguing questions come to mind: Do these interventions attenuate aging by interfering with common mechanisms? Do the mechanisms that are interfered with by CR, rapamycin, and RAS-bl mutually affect each other? Are there any pathways involved exclusively with a certain intervention?

Previously (101), we have discussed the converging effects displayed by CR and RAS-bl and summarized data showing that a number of the physiological benefits and molecular
events that occur in experimental CR and experimental and clinical RAS-bl involve changes in mitochondrial function. This observation is not unexpected considering both that aging is a process defined by a generalized time-related decline in physiological function (171), and mitochondria are crucial organelles engaged not only in cellular energy production, but also in the regulation of \( \text{Ca}^{2+} \) homeostasis (304), tissue \( \text{O}_2 \) gradients (397), apoptosis (44), and intracellular signaling (51). Furthermore, as a result of partial \( \text{O}_2 \) reduction by the mitochondrial respiratory chain, mitochondria are significant cellular sources of reactive oxygen species (ROS), and are themselves targets of ROS- and reactive nitrogen species (RNS)-mediated damage (52).

To address the questions posed above, we have structured the present review in the following order: 1) a brief update on the role of ROS and mitochondria in aging; 2) an introduction on mTOR, sirtuins, and KLOTHO as the most consistently altered targets in aging; 3) evidence in favor of RAS-bl, CR, and mTOR inhibition by rapamycin as interventions that extend lifespan; and 4) since CR is the most robust experimental intervention to retard aging, and accumulating data strongly support the key involvement of mTOR inhibition and the enhancement of sirtuin and KLOTHO signaling in the beneficial actions of CR in aging, we have summarized evidence linking RAS-bl to changes in mTOR, sirtuin, and KLOTHO activities.

It is worth mentioning that metformin exposure was also shown to prolong lifespan in Caenorhabditis elegans (50, 108, 308), rats (10), and mice (271). Metformin’s underlying life-extending mechanism is mostly unsettled and controversial, and is not further discussed here.

**Updating the Role of ROS and Mitochondria in Aging**

Mammalian aging can be described by its measurable consequences: the extents of medium and maximum lifespan, the structural and functional decline of particular tissues, a general decay in performance tests, and the presence of metabolic changes such as alterations in body composition, insulin resistance, and impairments in insulin-like growth factor-I (IGF-I), growth hormone, and sex steroid production (19).

Although aging is not a disease (330, 401), it is recognized as a process that augments the likelihood of disease emergence, particularly of those referred to as age-associated diseases such as hypertension, atherosclerosis and cardiovascular disease, type 2 diabetes, cancer, Alzheimer’s disease, osteoporosis, arthritis, cataracts, and neurodegenerative diseases, among others, whose incidences increase progressively with aging (43). Although both age-associated disease and the aging process are linked to derangements of the mTOR, sirtuin, KLOTHO, growth hormone (GH) releasing hormone-GH-IGF-I/insulin, p53, and apolipoprotein E pathways (15) they need to be distinguished as separate entities; however, interventions aimed at targeting the underlying aging process can potentially defer or interrupt the appearance of age-related disease.

Accumulation of ROS-mediated damage to cell proteins, lipids, carbohydrates, and nucleic acids is thought to be an underlying event in the aging process (24). The decisive contribution of mitochondria to the continuous production of ROS and RNS provides the foundation for the mitochondrial free radical theory of aging (285) as an extension of the more general free radical theory of aging (170). The mitochondrial free radical theory of aging proposes that the oxidation of mitochondrial components, mainly by mitochondrially derived-ROS (mtROS), alters mitochondrial function, which further intensifies mtROS production and macromolecule oxidation leading to the decline of cellular and organ function, which crucially contributes to establish lifespan and health span. Evidence in favor of the importance of reducing mitochondrial oxidative damage for the preservation of mitochondrial function and energy balance was obtained in mice with targeted mitochondrial overexpression of the human catalase gene (MCAT mice). MCAT mice were protected from age-associated structural and functional alterations in skeletal muscle mitochondria, accompanied with amelioration of lipid-induced muscle insulin resistance (242).

The involvement of ROS in the aging process has been variously tested by examining the effects of either exogenous antioxidant supplementation (reviewed in Ref. 346) or alterations in the genetic expression of antioxidant enzymes in mice (reviewed in Ref. 121). However, these approaches have produced conflicting results. Whereas in different model animals the modulation of antioxidant expression did not improve lifespan, it reduced the incidence of age-related diseases, pointing to oxidant damage as a serious influence on at least some facets of aging. The complex effects of exogenous antioxidant supplementation indicate that ROS are not exclusively prejudicial factors but also act as intracellular messengers that may promote favorable outcomes through hormetic responses (53, 367). In the same line, regarding an alternative aspect of mtROS, not long ago other reviews (261, 426) discussed abundant evidence supporting the notion that the regulation of aging and lifespan is crucially dependent on mitochondria-originated signaling, a process known as mitochondrial retrograde signaling (i.e., signaling from mitochondria to elsewhere in the cell). They also emphasized the beneficial and integrative roles of mild to moderate mtROS levels in the control of defensive, adaptive, and counteraging mechanisms, a process referred to as mitohormesis (340). The role of mitohormesis in longevity was analyzed in a very recent review (178); conversely, mitochondria can also deliver signals aimed at regulating innate immunity and systemic inflammation, indicating that they could sponsor inflammation in the course of the aging process (178). For an additional revision of current knowledge on the role of mitohormesis and mtROS in the promotion of health and lifespan, please refer to Ref. 339.

Finally, with the use of a different approach, a recent review that compared naturally long- and short-lived animals, and examined the effects associated to CR and genetic animal modification, also highlighted the relevance of mtROS as intracellular signaling agents for key cell functions and pointed out that mtROS involvement in aging most likely depends on the modulation of highly specific cellular actions and not on random oxidative injury to cell components (387).

Summarizing, it seems that ROS production, including mtROS, needs to be tightly controlled to avoid both the derangement of signaling cascades and oxidant damage.
mTOR, Sirtuins, and KLOTHO: The Most Consistently Altered Targets in Aging

mTOR functions. This section includes a condensed description of mTOR’s upstream and downstream signaling, intended to provide just enough background to reveal the potential pathways that CR, mTOR inhibition, and RAS-bl may share when interfering with aging.

mTOR is a highly conserved protein kinase whose mammalian version, mTOR, plays a role in human disease (237). mTOR is located mainly in the cytoplasm and lysosomes, but is also found in association with mitochondria, the plasma membrane, Golgi apparatus, endoplasmic reticulum, and nucleus (30). mTOR functions as the catalytic member of two structurally and functionally different protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (237). Both mTORC1 and mTORC2 regulate cell growth and metabolism. mTORC1 responds to extra- and intracellular signals; is activated by amino acids, growth factors, and oxygen; and is repressed by low cell energy levels (high AMP-to-ATP ratio) and stress conditions (energy deprivation, nutrient deprivation, hypoxia, heat shock). mTORC1 activation coordinate increases protein, lipid, and nucleotide synthesis; glycolysis; and cell growth and proliferation and reduces autophagy (236) (Fig. 1). Autophagy is a catabolic process that involves the degradation of dysfunctional or unneeded cell components, to preserve crucial cell activity and promote survival during nutrient limitation (220).

mTORC2 responds to hormones and growth factors such as insulin (31) and amino acids (395) and is sensitive to glucose deprivation or acute ATP depletion of cells (73). By modulating glucose metabolism, protein and lipid synthesis, and apoptosis, mTORC2 reorganizes the actin cytoskeleton, promotes cell enlargement, and regulates cell survival (reviewed in Refs. 113, 237, and 436) (Fig. 2).

Therefore, whereas mTORC1 regulates the timing of cell growth, spatial growth regulation is dependent on mTORC2. It follows that mTOR is crucially involved in tissue development (113, 237, and 436) (Fig. 2).

mTOR signaling: mTORC1 and mTORC2 molecular targets. Binding of ligands (such as insulin, amino acids, and proline-rich AKT substrate of 40 kDa (PRAS40) phosphorylation, which is thought to trigger PRAS40 dissociation from mTORC1 and alleviate its repressive influence on mTORC1 activity (reviewed in Refs. 236 and 237).

Cell energy depletion (high AMP-to-ATP ratio), including that prompted by mild hypoxia, activates AMP-activated protein kinase (AMPK), which phosphorylates and activates TSC1/2, thereby inhibiting mTORC1 (187, 258). Additionally, in response to energy deficiency, AMPK can diminish mTORC1 activity by direct phosphorylation of one of its composing proteins known as Raptor (161).

Low oxygen levels also modulate mTORC1 activity through hypoxia-related transcriptional activation of DNA-damage-inducible transcript 4 (also known as REDD1) that disrupts TSC2’s interaction with its inhibitory proteins, and allows TSC2 to block mTORC1 signaling (111).

The presence of intracellular amino acids signals to mTORC1 in a complex and still incompletely solved manner (122). Amino acid-initiated signaling drives mTORC1 from the cytosol to the lysosomal surface where it can be activated by Rheb (reviewed in Ref. 17). When present in its GTP-bound state, lysosomally localized Rheb positively regulates mTORC1 activity. In turn, Rheb is negatively controlled by the TSC2 component of TSC1/2, which converts GTP-bound Rheb into the inactive GDP-bound form (185). Amino acid-driven mTORC1 cytosol-to-lysosome translocation requires Rag GTPases (GTP-bound RagA/B and GDP-bound RagC), which act as the docking station for mTORC1 at this organelle. The RagA/B-RagC heterodimer stays bound to the lysosome thanks to its interaction with Ragulator (a trimeric protein complex); however, Ragulator also regulates Rag GTPase activity by functioning as a guanine nucleotide exchange factor for RagA/B (18). Furthermore, Ragulator interacts with lysosomal v-ATPase, and in the presence of amino acids both proteins undergo a conformational change that triggers Ragulator’s guanine nucleotide exchange factor activity toward RagA/B, thereby activating it (18). In addition, amino acids signal for mTORC1 activation by stimulating folliculin GTPase activity, which promotes RagC GTP hydrolysis at the lysosomal surface (408). When amino acid levels are low, the octomeric complex GAP activity towards Rags (GATOR) negatively regulates Rag GTPases by means of the GTPase activating activity of its GATOR1 subcomplex, which inactivates RagA/B, leading to mTORC1 silencing. When amino acid levels are high, the GATOR2 subcomplex is able to inhibit GATOR1, which contributes to mTORC1 activation (16). The Sestrins add a further level of control over mTORC1 activity; thus, when amino acids are scarce, Sestrins 1 to 3 interact with GATOR2 to negatively regulate mTORC1 signaling (70). Recent data point to the lysosomal transmembrane protein SLC38A9, an amino acid transporter, as an arginine sensor that interacts with Ragulator and Rag GTPases to positively regulate mTORC1 (333, 427).

By furnishing carbons to the mitochondrial tricarboxylic acid (TCA) cycle to generate ATP, glucose and glutamine positively signal to mTORC1, independently of AMPK, TSC1/2, and Rag-proteins, by engaging the TTT-RUVBL1/2 complex that regulates mTORC1 assembly and lysosomal localization (216).

mTORC1 positively regulates protein synthesis mainly by phosphorylating the p70 ribosomal S6 kinase (p70-S6K or S6K1) and the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1). mTORC1-mediated activation of S6K1 regulates the activity of many downstream proteins, resulting in increased mRNA synthesis, translation...
Fig. 1. Mechanistic target of rapamycin complex 1 (mTORC1) activation and its cellular consequences. mTORC1 integrates intra- and extracellular signals, including amino acids, growth factors, energy status, oxygen and stress mediators. Binding of ligands (such as insulin) to their specific growth factor receptors located at the cell surface triggers a signaling cascade that activates phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), Akt, extracellular signal-regulated kinase 1/2 (Erk1/2) and S6K. As a result of this, Erk1/2 and S6K phosphorylate and inhibit the tuberous sclerosis 2 (TSC2)-TSC1 protein complex, thereby releasing its negative regulatory action on mTORC1 and leading to mTORC1 activation. Akt also directly activates mTORC1 through PRAS40 phosphorylation. Cell energy depletion (high AMP-to-ATP ratio) activates AMP-activated protein kinase (AMPK), which phosphorylates TSC2, thereby reducing mTORC1 activation. Also, in response to energy deficiency, AMPK can diminish mTORC1 activity by direct phosphorylation of one of its composing proteins, known as Raptor. The presence of intracellular amino acids signals to mTORC1 in a complex and still incompletely solved manner that includes the participation of Ragulator, v-ATPase, Rag A/B, Rag C/D, GATOR 1/2, Sestrins 1/2/3, folliculin, and SLC38A9. When activated, mTORC1 promotes cell growth and proliferation by stimulating protein, lipid, and organelle biosynthesis and by inhibiting autophagy. mTORC1 positively regulates protein synthesis mainly by phosphorylating the p70 ribosomal S6 kinase (p70-S6K or S6K1) and the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1). mTORC1-mediated activation of S6K1 regulates the activity of many downstream proteins, resulting in increased mRNA synthesis, translation and elongation, and ribosomal protein synthesis. The phosphorylation of 4E-BP1 prevents it from binding eIF4E, and allows the latter to support cap-dependent translation. mTOR inhibits autophagy by phosphorylating and consequently repressing unc-51-like kinase 1 (ULK1) and autophagy-related gene 13 (ATG13), which together with focal adhesion kinase family-integrating protein of 200 kDa (FIP200) form a protein complex that induces autophagy when activated. Activation of mTORC1 positively regulates de novo lipid synthesis by activating sterol regulatory element binding protein 1 (SREBP1) and peroxisome proliferator-activated receptor-γ (PPAR-γ), which in turn promote the expression of genes coding for lipid and cholesterol homeostasis proteins. Continuous arrows indicate direct positive regulatory actions, dashed arrows represent positive regulatory actions that include intervening steps, and blunt-ended lines indicate inhibitory effects.
and elongation, and ribosomal protein synthesis. The phosphorylation of 4E-BP1 prevents it from binding eIF4E and allows the latter to support cap-dependent translation. Also, in the presence of growth factors and nutrients, mTOR inhibits autophagy by phosphorylating Ser757 and consequently repressing unc-51-like kinase 1 (ULK1) (213). However, when nutrients are scarce, AMPK directly phosphorylates Ser317 and Ser777 and activates ULK1, thereby stimulating autophagy (213).

Activation of mTORC1 positively regulates de novo lipid synthesis by activating both esterol regulatory element-binding protein 1 and peroxisome proliferator-activated receptor-γ (PPAR-γ), which in turn promote the expression of genes coding for lipid and cholesterol homeostasis proteins (93, 236, 237). PPARs are nuclear hormone receptors that belong to a superfamily of transcription factors involved in the regulation of lipid and carbohydrate metabolism, and inflammation.

Under normoxic conditions, mTORC1 upregulates hypoxia-inducible factor 1 (HIF1) expression in a PI3K- and AKT-independent manner; this mTORC1-HIF1 pathway is involved in maintaining glucose metabolism and glycolysis (120, 136).

**mTORC2.** Apart from responding to glucose, hormones, growth factors, and amino acids; cell ATP levels control mTORC2's basal kinase activity, which preserves mTORC2 complex integrity and Akt phosphorylation. Activated mTORC2 phosphorylates and activates Akt, which in turn phosphorylates and inhibits the forkhead box O1 (FOXO1) and FOXO3 transcription factors. In response to cellular stress, FOXOs promote cell growth inhibition and/or apoptosis by inducing the expression of proapoptotic mitochondria-targeting proteins, death receptor ligands, or cyclin-dependent kinase inhibitors. Inhibition of FOXOs transcriptionsal activities by Akt promotes cell survival, growth, and proliferation. mTORC2-dependent activation of PKC-α, paxillin (an actin filament regulatory protein), and Rho GTPases, among others, affects the actin cytoskeleton dynamics and regulates cell shape. mTORC2 also activates SGK1, thereby promoting ion transport and cell growth; also, mTORC2 may influence hepatic metabolism by activating p38MAPK signaling. mTORC2 is considered to be upstream of mTORC1, since activated mTORC2 phosphorylates Akt, which in turn phosphorylates and inhibits TSC2, thereby releasing the latter’s suppressor action over mTORC1 or PRAS40. mTORC1 activation triggers the phosphorylation of one of mTORC2 protein components (Rictor), which inhibits mTORC2 signaling. Continuous arrows indicate direct positive regulatory actions, dashed arrows represent positive regulatory actions that include intervening steps, and blunt-ended lines indicate inhibitory effects.

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Fig. 2. Mechanistic target of rapamycin complex 2 (mTORC2) activation and its cellular consequences. mTORC2 responds to glucose, hormones, growth factors, and amino acids; cell ATP levels control mTORC2's basal kinase activity, which preserves mTORC2 complex integrity and Akt phosphorylation. Activated mTORC2 phosphorylates and activates Akt, which in turn phosphorylates and inhibits the forkhead box O1 (FOXO1) and FOXO3 transcription factors. In response to cellular stress, FOXOs promote cell growth inhibition and/or apoptosis by inducing the expression of proapoptotic mitochondria-targeting proteins, death receptor ligands, or cyclin-dependent kinase inhibitors. Inhibition of FOXOs transcriptionsal activities by Akt promotes cell survival, growth, and proliferation. mTORC2-dependent activation of PKC-α, paxillin (an actin filament regulatory protein), and Rho GTPases, among others, affects the actin cytoskeleton dynamics and regulates cell shape. mTORC2 also activates SGK1, thereby promoting ion transport and cell growth; also, mTORC2 may influence hepatic metabolism by activating p38MAPK signaling. mTORC2 is considered to be upstream of mTORC1, since activated mTORC2 phosphorylates Akt, which in turn phosphorylates and inhibits TSC2, thereby releasing the latter’s suppressor action over mTORC1 or PRAS40. mTORC1 activation triggers the phosphorylation of one of mTORC2 protein components (Rictor), which inhibits mTORC2 signaling. Continuous arrows indicate direct positive regulatory actions, dashed arrows represent positive regulatory actions that include intervening steps, and blunt-ended lines indicate inhibitory effects.

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<td>Growth factors / hormones</td>
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<td>Amino acids</td>
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<td>Glucose</td>
<td>mTORC2</td>
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**Deprotor**

mTORC2 UPSTREAM SIGNALING

**ACTIVE mTORC2**

**mTORC2 DOWNSTREAM SIGNALING**

**INITIAL RESPONSES**

**FINAL RESPONSE**

**CELL GROWTH AND SURVIVAL**

**CYTOSKELETON ORGANIZATION**

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**Review**

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**Fig. 2.** Mechanistic target of rapamycin complex 2 (mTORC2) activation and its cellular consequences. mTORC2 responds to glucose, hormones, growth factors, and amino acids; cell ATP levels control mTORC2's basal kinase activity, which preserves mTORC2 complex integrity and Akt phosphorylation. Activated mTORC2 phosphorylates and activates Akt, which in turn phosphorylates and inhibits the forkhead box O1 (FOXO1) and FOXO3 transcription factors. In response to cellular stress, FOXOs promote cell growth inhibition and/or apoptosis by inducing the expression of proapoptotic mitochondria-targeting proteins, death receptor ligands, or cyclin-dependent kinase inhibitors. Inhibition of FOXOs transcriptional activities by Akt promotes cell survival, growth, and proliferation. mTORC2-dependent activation of PKC-α, paxillin (an actin filament regulatory protein), and Rho GTPases, among others, affects the actin cytoskeleton dynamics and regulates cell shape. mTORC2 also activates SGK1, thereby promoting ion transport and cell growth; also, mTORC2 may influence hepatic metabolism by activating p38MAPK signaling. mTORC2 is considered to be upstream of mTORC1, since activated mTORC2 phosphorylates Akt, which in turn phosphorylates and inhibits TSC2, thereby releasing the latter’s suppressor action over mTORC1 or PRAS40. mTORC1 activation triggers the phosphorylation of one of mTORC2 protein components (Rictor), which inhibits mTORC2 signaling. Continuous arrows indicate direct positive regulatory actions, dashed arrows represent positive regulatory actions that include intervening steps, and blunt-ended lines indicate inhibitory effects.

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Activated mTORC2 phosphorylates and activates AKT, which in turn phosphorylates and inhibits the forkhead box O1 (FOXO1) and FOXO3 transcription factors. In response to cellular stress, FOXOs promote cell growth inhibition and/or apoptosis by inducing the expression of proapoptotic mitochandria-targeting proteins, death receptor ligands, or cyclin-dependent kinase inhibitors (45, 149, 273, 406, 452) (Fig. 2). Therefore, inhibition of FOXO transcriptional activities by AKT promotes cell survival, growth, and proliferation. In addition, in cultured neuronal cells and fibroblasts, FOXO3a activation provides protection against oxidative stress by up-regulating the transcription of manganese superoxide dismutase (MnSOD) and catalase (223, 302).

mTORC2-dependent activation of protein kinase C (PKC)-α, paxillin (an actin filament regulatory protein), and Rho GTPases, among others, affects the actin cytoskeleton dynamics and regulates cell shape (3, 191, 357).

Another known target of mTORC2 is the kinase serum- and glucocorticoid-induced protein kinase 1 (SGK1), whose activation promotes ion transport and cell growth (145, 175). Recent data suggest that mTORC2 may influence hepatic metabolism by activating p38 protein (p38) mitogen-activated protein kinase (MAPK) signaling (232).

Finally, mTORC2 is considered to be upstream of mTORC1, since activated mTORC2 phosphorylates AKT, which in turn phosphorylates and inhibits TSC2 thereby releasing the latter’s suppressor action over mTORC1 (186, 267, 412) or PRAS40 (414). To further complicate the interrelations between mTORC1 and mTORC2, mTORC1 activation triggers the phosphorylation of one of mTORC2 protein components (Rictor), which inhibits mTORC2 signaling (197); also, mTORC1 negatively impacts on mTORC2 activity via the phosphorylation of insulin receptor substrate 1 (IRS1) (371, 410) and growth factor receptor-bound protein 10 (181, 446), and alternatively that of SIN1 (also known as mitogen-activated protein kinase-associated protein 1) (259, 260).

**Consequences of mTOR inhibition: The extension of lifespan.** Rapamycin, a potent antiproliferative and immunosuppressant agent produced by *Streptomyces hygroscopicus*, inhibits several mTORC1 functions after complexing with the FK506-binding proteins (FKBP12) and binding mTORC1 (59). Although mTORC2 was originally thought to be rapamycin-insensitive (191), in some cell types (316) and in mice in vivo (233) long-term rapamycin treatment diminishes mTORC2 signaling. Rapamycin does not bind mTORC2, although it seems to inhibit mTORC2 assembly in a FKBP12-dependent way (358). The mechanism responsible for the differential cell response of mTORC2 to rapamycin is not well understood; however, recent data showed that whether a tissue or a cell line responds to rapamycin by inhibiting mTORC2 relies on the relative expression of the FK506-binding proteins FKBP12 and FKBP51 (366).

Inhibition of TOR activity extended lifespan in yeast, worms, and flies (reviewed in Ref. 206).

In mice, late-start [20-mo-old (173) and 9-mo-old (281)] rapamycin-mediated mTOR inhibition increased medium and maximal lifespan but did not affect the cause of death or disease distribution, suggesting that rapamycin may expand lifespan by retarding age-associated disease. Present knowledge regarding the effects of pharmacological or genetic mTOR blockade on mammalian aging and lifespan was reviewed in Ref. 234.

Of note, the effects of rapamycin on rodent longevity (282), as well as the consequences of genetic interventions that modify the insulin/IGF-I/mTOR pathway (233, 369, 370), are invariably more favorable in females than in males (discussed in Ref. 231).

Importantly, in humans, molecular epidemiological data in two independent study cohorts revealed that in older individuals gene expression changes are reconcilable with global downregulation of mTOR-related transcripts (S6K, SREBF1, FOXO, phosphatidylinositol 3,4,5-triphosphate 3-phosphate, PI3K, pyruvate dehydrogenase kinase 1, SGK1, vascular endothelial growth factor B, hypoxia-inducible factor 1A, PKC); also, the changes were largely comparable to those observed in experimental mTOR inhibition and the related lifespan increase in animals (172).

Accordingly, in cultured primary human fibroblasts, senescence is associated with age-related changes in the expression of mTORC1 transcripts, whereas in human endothelial cells the response is more limited and comprises mTORC2-related transcripts (127).

Because mTORC1 activation by nutrients and growth factors promotes protein synthesis and inhibits autophagy, it was suggested that prolonged activity in time may result in protein aggregation, excess oxidant levels, and cell and tissue damage, thereby prompting the progression of age-related disease (Fig. 3). Thus, rapamycin and CR (for the relation between CR and mTOR, see *Calorie restriction and aging, mTOR, and AMPK*) may extend lifespan and retard aging by interfering...
with the above processes downstream of mTORC1 (237). Age-related myocardial hypertrophy is known to be driven by increased protein synthesis; importantly, rapamycin prevents, and also reverses, myocardial hypertrophy in rodents (428), indicating that mTOR is involved in the process. Supported by recent work (64, 71, 72, 442), another interesting possibility to explain the mechanism(s) involved in mammalian lifespan extension by mTORC1 inhibition is that mTORC1 can prevent tissue decay by ameliorating stem cell function.

However, the issue of whether the lifespan-extending effects of rapamycin may result from rapamycin’s actions on particular life-shortening diseases, such as cancers, rather than slowing the rate of aging itself, remains largely unsettled. This issue was recently addressed by comprehensively evaluating a wide spectrum of structural and functional aging phenotypes in male C57BL/6J mice (301). Rapamycin increased mice lifespan, but it improved only a minority of the studied aging phenotypes and relieved a subgroup of aging characteristics. Nonetheless, rapamycin also improved many of these characteristics in young mice, pointing to drug actions that do not depend on aging. These data support the concept that rapamycin’s longevity actions result from its effects on particular life-shortening diseases, such as cancers (that are the dominant cause of death in abundant mouse strains), rather than from slowing the rate of aging. In genetically heterogeneous mice, rapamycin treatment beginning at 9 mo of age significantly extended lifespan, and in 7% of the control mice vs. 11% of the rapamycin mice the probable cause of death was a nonneoplastic disease (281).

Studies aimed at assessing cardiovascular mTOR/S6K1 activity in aging rodent models yielded contradictory results. Thus, cardiac microarray data revealed that mTOR-related gene expression is reduced upon aging in Fischer rats (256), suggesting an age-associated downregulation of mTOR signaling; contrarily, basal mTOR-mediated phosphorylation of S6K1 was increased in aortas from Fischer × Brown Norway F1 hybrid (336), which suggests the augmentation of mTOR/S6K1 signaling in aging.

Finally, evidence indicating that mTOR-derived signals from one tissue can also impact on other organs and, as a result, can influence overall body metabolism and energy homeostasis in a noncell autonomous way has been recently reviewed (7). In this context, the authors pointed out that it is not astonishing that mTOR-derived signals are involved in aging and are frequently deranged in metabolic conditions, such as diabetes, obesity, and cancer.

mTOR and mitochondria. In cultured leukemia cells, disruption of mTORC1 activity using either rapamycin or RNA interference decreased both basal oxygen consumption and oxidative capacity, independent of mTORC1’s cellular targets S6K1 and 4EBP1 (363).

In mouse skeletal muscle and in cultured myoblasts, rapamycin-mediated mTOR inhibition decreased the expression of three mitochondrial transcription regulators, i.e., peroxisome proliferator activator receptor-γ coactivator-1α (PGC-1α), nuclear respiratory factors (NRF), and estrogen-related receptor-α, which resulted in reduced expression of mitochondrial genes and oxygen consumption (89, 440). PGC-1α coactivates several transcription factors, thereby coordinating the expression of the mitochondrial and nuclear genomes into a program of mitochondrial biogenesis, and also controls the expression of genes involved in lipid oxidation (418); NRFs are transcription factors that regulate the expression of nuclear genes for mitochondrial respiratory chain proteins (360); and estrogen-related receptor-α is required for mitochondrial gene activation and enhanced mitochondrial biogenesis (96).

Also, in mouse skeletal muscle, mTOR was found to control the expression of mitochondrial genes after forming a complex with the transcription factor Yin Yang 1 (YY1) and PGC-1α, and YY1 knockdown resulted in decreased mitochondrial respiration and gene expression (89). This evidence points to mTOR as a necessary regulator of energy status mediated by transcriptional control of mitochondrial oxidative function. The role of mTORC1 and YY1 in the maintenance of mitochondrial function was further revealed in work showing that skeletal muscle-specific YY1 knockout mice display seriously abnormal mitochondrial oxidative function and morphology, and that mTOR promotes the interaction between YY1 and PGC-1α by phosphorylating YY1 (34).

Direct evidence of mTOR control over mitochondrial function was found in leukemic cells, where rapamycin treatment reduced mitochondrial function, thus favoring aerobic glycolysis over mitochondrial respiration. Furthermore, mTOR regulates mitochondrial metabolism by phosphorylating B cell lymphoma-extra large (Bcl-xl) and thereby controlling Bcl-xl’s association with mTOR, both of which form a complex with voltage-dependent anion channel selective protein 1 at the outer mitochondrial membrane (328).

In cultured cells and in mice, mTORC1 controls mitochondrial function and biogenesis via the inhibition of 4E-BP1 and 4E-BP2 and the ensuing selective translation activation of nuclear mRNAs for mitochondrial function proteins, including several ATP synthase subunits, mitochondrial transcription factor A, mitochondrial ribosomal proteins, and NADH dehydrogenase-1α subcomplex assembly factors 2 and 4 (293). Because mRNA translation is considered the largest cell energy spending process, and experimental constitutive mTORC1 activation stimulates mitochondrial function and ATP production in a translation-dependent manner, it has been proposed that mTORC1 is a key element in a feedforward circuit that connects mRNA translation to oxidative phosphorylation (293).

Finally, there is evidence linking mTORC2 to mitochondrial function (reviewed in Ref. 30).

These observations suggest that, despite enhancing mice longevity, rapamycin treatment might be debilitating or predispose to the collapse of energy production. However, the doses of rapamycin that can prolong mice lifespan did not induce manifest mitochondrial dysfunction in mouse skeletal muscle (441). In this scenario, recent evidence in mice is consistent with the concept that the increase of lifespan brought about by rapamycin depends on the energy-saving reductions of protein synthesis and cell proliferation, which might assign the resulting surplus energy to the preservation of mitochondrial proteins, among other pivotal proteins (116). Both in males and females, skeletal muscle mitochondrial protein synthesis and global cardiac protein synthesis were maintained in rapamycin-treated animals despite decreased DNA synthesis, and ribosomal protein S6 phosphorylation; in the liver and heart, male response to rapamycin administration was more robust than that in females.

A recent review (432) discusses the role played by mitochondria in the regulation of longevity by mTOR, including...
mTOR’s involvement in apoptosis, mitochondrial biogenesis and metabolism (mitochondrial membrane potential, O$_2$ consumption, ATP production), mitophagy, and mitochondrial hormesis. The authors also discuss the concept that failure of mitochondrial adaptive responses in preference to free radicals as such may contribute to the aging process.

**Sirtuins.** From bacteria to humans, sirtuins are highly conserved NAD-dependent deacetylases, and the requirement for NAD$^+$ in sirtuin activation provides a link between metabolism and aging/age-related disease. Seven sirtuins (SIRT1-7) with different cell functions and distributions were described in mammals. The most widely studied member of the family, SIRT1, enhances transcriptional repression and chromatin silencing by deacetylating histone; also, by deacetylating transcription factors and coregulatory proteins, it modulates numerous signaling and survival pathways. Thus, in response to cell energy and redox status, SIRT1 regulates cell survival, mitochondrial biogenesis, metabolism, and stress responses, among others (reviewed in Ref. 297). It follows that SIRT1 deficiency brought about by stress, i.e., hypoxia, oxidative, or metabolic derangements, can promote age-associated disease, including renal and cardiovascular disease, neurodegeneration, and diabetes. Also, brain-specific SIRT1 overexpression increases lifespan and retards aging in mice (359). Cytosolic SIRT2 is involved in cell cycle regulation (118) and was shown to extend lifespan of the long-lived BubR1 mice that overexpress the mitotic checkpoint kinase gene BubR1 (306); SIRT3, -4, and -5 are localized to the mitochondria, where SIRT3 and SIRT5 modulate the mitochondrial urea cycle and fatty acid oxidation (166, 298), whereas SIRT4 is an ADP-ribosyltransferase that regulates insulin secretion (4). SIRT6 is a nuclear protein that contributes to DNA repair (202) and seems to be involved in inflammation (209), and nuclear SIRT7 promotes ribosomal DNA transcription (158). Although SIRT4, SIRT5, and SIRT7 are involved in cytoprotection, little is known about their molecular targets and biological actions (297). In addition, SIRT3 and SIRT6 are engaged in age-related disease and longevity (203, 219) (see The role of sirtuins in CR).

The sirtuin-mitochondria connection. Regarding the relation between sirtuins and mitochondria, SIRT1 activation stimulates mitochondrial biogenesis (11), and SIRT3 reduces oxidative damage in CR by improving the mitochondrial glutathione antioxidant system (379) and activating the mitochondrial MnSOD by direct enzyme deacetylation (324). A recent review (39) discussed how SIRT1 and SIRT3 can cooperate to control mitochondrial biogenesis.

Also, in cultured mouse cells, SIRT3 acted downstream of PGC-1α in mediating the induction of antioxidant enzymes and certain respiratory chain components (222).

Other mitochondrial metabolic targets activated by SIRT3 and SIRT5 include enzymes involved in fatty acid oxidation (179), acetate (165), and urea metabolism (166, 298). Of note, contrarily to what happens with other sirtuins, the expression of mitochondrial SIRT4 is reduced by CR (163). This is not surprising since normally SIRT4 inhibits glutamate dehydrogenase activity leading to decreased α-ketoglutarate production; when SIRT4 is downregulated by CR, glutamate dehydrogenase activity raises, which increases α-ketoglutarate production allowing it to enter the TCA cycle and furnish energy during CR.

Finally, SIRT5 participates in the regulation of mitochondrial energy metabolism by acting downstream of PGC-1α and AMPK (that promote opposed actions on SIRT5 expression) in mouse hepatocytes (46). SIRT5 is a desuccinylase that selectively eliminates specific succinyl residues from a variety of proteins, including those involved in oxidative phosphorylation, fatty acid oxidation, and production of ketone bodies (329). This is consistent with the observed increases in ATP synthesis and oxygen consumption in human hepatocellular carcinoma (Hep G2) cells that overexpress SIRT5, which was not accompanied with enhanced mitochondrial biogenesis (46).

Cross talk between mTOR and sirtuin. mTOR, by acting as a sensor of extra- and intracellular stimuli, allows to shuttle between anabolism and catabolism to permit cell adaptation to nutrient availability. When the cellular AMP-to-ATP ratio increases AMPK is activated, which leads to mTOR inactivation and the consequent saving of cell energy for essential functions.

Based on their observation that yeast lifespan extension induced by CR is mediated by TOR inhibition and sirtuins acting in the same pathway Medvedik et al. (277) proposed that in mammals mTOR signaling and sirtuins may also function in the same longevity pathway. In support of this view, resveratrol, a plant-derived polyphenol that activates sirtuins, inhibits rat cardiac myocyte hypertrophy by activating AMPK (68) and reducing the activities of ribosomal S6 kinase (S6K) and eEF2 (a translation elongation factor) both of which are regulated by mTOR signaling.

Of note, AMPK, SIRT1, and SIRT3 are pivotal energy status sensors that participate in the regulation of glucose and lipid metabolism (57). They cooperate with PGC-1α to modulate mitochondrial metabolism, proliferation, and energy consumption (39).

Also, mTORC1 can phosphorylate and activate S6K, only after S6K has been deacetylated by SIRT1/SIRT2 (180); thus, although S6K1 activity is closely linked to its mTORC1-dependent phosphorylation state, an additional sirtuin-mediated regulatory modification contributes to mTORC1-dependent S6K1 activation.

Finally, in cultured mouse embryonic fibroblasts and in HeLa cells, SIRT1 inhibits mTOR signaling, apparently through TSC2 (150).

**KLOTHO/FGF23 and vitamin D.** KLOTHO, which exists both as a membrane- and a soluble-secreted protein, is primarily expressed in the kidney, parathyroid gland, brain choroid plexus, and skeletal muscle (249). Transmembrane KLOTHO functions as a coreceptor for fibroblast growth factor 23 (FGF23), an obligatory step that allows high-affinity FGF23 binding to its receptors (411). Activation of KLOTHO/FGF23 promotes phosphaturia by inhibiting the Na-dependent phosphate cotransporters (NaPi-IIa) in the brush border of kidney tubular cells (272).

Soluble KLOTHO has a glycosidase activity that, by altering the sugar composition of certain proteins, regulates their activity and/or docking time at the cell surface. In this way soluble KLOTHO regulates various ion channels and transporters (184), and growth factor signaling [including Wnt, IGF-I, and transforming growth factor (TGF)-β1] (257, 426). In distal convoluted tubules, KLOTHO’s β-glycosidase function captures TRPV5 Ca$^{2+}$ channels in the plasma membrane, maintaining renal Ca$^{2+}$ permeability (69, 265). In addition, soluble KLOTHO protects cells from oxidative stress (226) and di-
rectly regulates phosphate transport by deglycosylating NaPi-IIa cotransporters in the kidney proximal tubule (183).

Apart from suppressing phosphate reabsorption, KLOTHO/FGF23 blunt renal 1,25-dihydroxyvitamin D [1,25-(OH)2D3; the active vitamin D metabolite] synthesis by inhibiting 1α-hydroxylase in the kidney, which leads to the decline of circulating 1,25-(OH)2D3 content (444) (Fig. 4); also, KLOTHO/FGF23 signaling reduces parathyroid hormone (PTH) production and secretion, further abolishing renal 1,25-(OH)2D3 synthesis (25, 224, 270). FGF23 also stimulates active 1,25-(OH)2D3 degradation by inducing renal cytochrome P-450, family 24, subfamily A, polypeptide 1 (CYP24A1), the enzyme that degrades 25-hydroxyvitamin D and 1,25-(OH)2D3 (374).

Therefore, FGF23 is a phosphaturic agent that controls phosphate balance directly in the kidney (325), and indirectly by offsetting 1,25-(OH)2D3 actions on intestinal sodium-dependent phosphate cotransporters (80). However, 1,25-(OH)2D3 and KLOTHO/FGF23 maintain a reciprocal interaction, since 1,25-(OH)2D3 stimulates the expression of FGF23 and KLOTHO by binding to a vitamin D response element in the promoter region of each gene (142, 318) (Fig. 4).

Summarizing, as a result of its multiple actions, KLOTHO contributes to control Ca2+ phosphate, and vitamin D metabolism in the kidney.

Also, in the mutant klotho (kl/kl) mouse model of accelerated aging, transgenic KLOTHO overexpression extended lifespan by inhibiting insulin and IGF-I signaling (376), whereas intraperitoneal administration of soluble KLOTHO attenuated renal fibrosis by downregulating TGF-β signaling (77).

Accumulating evidence points to KLOTHO, FGF23, and vitamin D as interactive elements that compose an endocrine axis for phosphate and Ca2+ metabolism, whose disturbance is implicated in renal disease progression (97). KLOTHO seems to function collaboratively with PTH on Ca2+ and phosphate transport, although it opposes PTH’s positive action on renal 1,25-(OH)2D3 synthesis.

FGF23, KLOTHO, and vitamin D in vivo interactions have been recently reviewed (331).

Phosphate and Ca2+2+; Their role in aging. Regarding the role of Ca2+ dysregulation in the aging process, depletion of FGF23/KLOTHO function in mice and humans is associated with accelerated aging, soft tissue calcification, and higher mortality, all of which may result from hyperphosphatemia and hypercalcemia. KLOTHO absence, or detrimental actions of increased FGF23 or 1,25-(OH)2D3 levels. Thus, mice that lack either FGF23 (332) or KLOTHO not only display hyperphosphatemia but also develop various age-related derangements (growth deceleration, reduced lifespan, muscle and skin atrophy, vascular calcification, hypoglycemia, hypervitaminosis D, hypercalcemia, osteoporosis, hypogonadism, emphysema, and premature involution of the thymus, among others), which are relieved by lowering phosphate to normal levels (reviewed in Ref. 228). A reversal of early age-related changes in Fgf23−/− mice is also found when 1,25-(OH)2D3 activity is removed by deleting the 1α-hydroxylase gene (332). However, other evidence showed that when hyperphosphatemia and excess 1,25-(OH)2D3 activity were independently normalized by feeding the FGF23-null mice either a phosphate- or vitamin D-deficient diet, only the low-phosphate diet completely reversed the Fgf23−/− mice phenotype, although serum 1,25-(OH)2D3 and

![Fig. 4. KLOTHO, fibroblast growth factor 23 (FGF23), vitamin D, and renin-angiotensin system (RAS) interactions. Klotho and FGF-23 inhibit renal 1,25-dihydroxyvitamin-D [1,25-(OH)2D3; the active vitamin-D metabolite] synthesis and reduce parathyroid hormone (PTH) production and secretion, further abolishing renal 1,25-(OH)2D3 synthesis. FGF23 also stimulates active 1,25-(OH)2D3 degradation by inducing renal CYP24A1, the enzyme that degrades 25-hydroxyvitamin-D and 1,25-(OH)2D3. However, 1,25-(OH)2D3 and Klotho/FGF23 maintain a reciprocal interaction, since 1,25-(OH)2D3 stimulates the expression of FGF23 and Klotho by binding to a VDRE in the promoter region of each gene. After binding the VDR, 1,25-(OH)2D3 inhibits kidney renin gene expression by interfering with the formation of a transcriptional complex that binds the renin gene promoter; also, 1,25-(OH)2D3 downregulates AT1R expression. Angiotensin II reduces renal Klotho expression, which interferes with FGF23 signaling and results in elevated FGF23 levels. In turn, the increased FGF23 content inhibits 1α-hydroxylase, leading to the lowering of 1,25-(OH)2D3 production.](http://ajpheart.physiology.org/)

**RENNIN ANGIOTENSIN SYSTEM**

- Angiotensinogen
- Renin
- Angiotensin I (ACE)
- Angiotensin II

**VITAMIN D-FGF23-KLOTHO AXIS**

- 25-hydroxyvitamin D
- 1α-hydroxylase
- 1,25-dihydroxyvitamin D
- Kidney Parathyroid
- Klotho
- FGF23

*Fig. 4. KLOTHO, fibroblast growth factor 23 (FGF23), vitamin D, and renin-angiotensin system (RAS) interactions. Klotho and FGF-23 inhibit renal 1,25-dihydroxyvitamin-D [1,25-(OH)2D3; the active vitamin-D metabolite] synthesis and reduce parathyroid hormone (PTH) production and secretion, further abolishing renal 1,25-(OH)2D3 synthesis. FGF23 also stimulates active 1,25-(OH)2D3 degradation by inducing renal CYP24A1, the enzyme that degrades 25-hydroxyvitamin-D and 1,25-(OH)2D3. However, 1,25-(OH)2D3 and Klotho/FGF23 maintain a reciprocal interaction, since 1,25-(OH)2D3 stimulates the expression of FGF23 and Klotho by binding to a VDRE in the promoter region of each gene. After binding the VDR, 1,25-(OH)2D3 inhibits kidney renin gene expression by interfering with the formation of a transcriptional complex that binds the renin gene promoter; also, 1,25-(OH)2D3 downregulates AT1R expression. Angiotensin II reduces renal Klotho expression, which interferes with FGF23 signaling and results in elevated FGF23 levels. In turn, the increased FGF23 content inhibits 1α-hydroxylase, leading to the lowering of 1,25-(OH)2D3 production.*
Ca\(^{2+}\) contents continued to be elevated. Normalization of serum 1,25-(OH\(_2\))D\(_3\) levels was accompanied with enduring hypophosphatemia, extensive vascular calcifications, and increased survival in the FGF23 null phenotype. These observations suggest that hyperphosphatemia instead of excess 1,25-(OH\(_2\))D\(_3\) is mainly responsible for vascular calcifications in FGF23-null mice and that excess 1,25-(OH\(_2\))D\(_3\) can also be detrimental when accompanied with hyperphosphatemia and FGF23 absence (388).

Inorganic phosphate (Pi) is a crucial element in the composition of cellular biomolecules, such as phospholipids and nucleic acids, and in various cell functions, including cellular signaling and energy production. As a result, serum phosphate dysregulation participates in the development of multiple pathological derangements such as vascular calcification, bone conditions, cancer, and chronic kidney disease (54, 227). How Pi directly affects aging is largely unknown; however, Pi was shown to increase oxidative stress in vitro and in vivo and to influence glucose metabolism and insulin sensitivity (reviewed in Ref. 227), both of which have been suggested to impact on age-related changes across species. Relating to the role of Pi in vascular calcification, studies in vitro and in animal models indicate that high Pi levels (consistent with those present in hyperphosphatemic patients) directly promote the osteogenic conversion of smooth muscle cells and contribute to the formation of hydroxyapatite crystals (123, 250, 303, 347).

An approach that combined transcriptomics and proteomics analyses of preosteoblasts and primary mouse and human mesenchymal stromal cells revealed that high phosphate levels have a mitogenic effect and delineated the mechanisms involved in phosphate-mediated stimulation of cell proliferation and matrix regulation (54).

In humans, altered phosphate metabolism is associated with age-related changes, inflammation, and death in chronic kidney disease (190, 300), and in young healthy subjects serum phosphorus content is associated with coronary atherosclerosis later in life (139).

The above findings expose unexpected relations between phosphate metabolism and aging, where KLOTHO, FGF23, and vitamin D are pivotal players.

**Phosphate and mitochondria.** Interestingly, Pi is involved in the regulation of mitochondrial function. Once inside the cell, Pi is transported into the mitochondria to act as an ATP synthase substrate and a modulator of oxidative phosphorylation. Thus, in isolated porcine heart and skeletal muscle mitochondria, oxygen consumption, mitochondrial membrane potential, and NADH content were progressively stimulated by increasing extramitochondrial phosphate levels, indicating that Pi regulates oxidative phosphorylation at different points (37). Of note, mtROS production is positively related to mitochondrial membrane potential (310). In addition, elevated Pi levels intensified the flux of reducing equivalents toward Complex III’s cytochrome c, which is known to increase ROS generation (37). As a whole, the above evidence indicates that high phosphate alters mitochondrial function, thereby increasing mtROS production. Further work showed that Pi activates the opening of the mitochondrial permeability transition pore, a nonselective inner mitochondrial membrane pore, that mediates necrotic cell death in cells under ischemia-reperfusion stress (416).

Finally, as pointed out by Kuro-o (227) the concept that Pi is involved in the aging process is supported by the observed inverse relation between lifespan and serum phosphate levels in diverse mammalian species, from rodents to humans (\(r^2 = 0.8942\)).

**Vitamin D and aging.** By binding the nuclear vitamin D receptor (VDR), 1,25(OH\(_2\))D\(_3\) participates in the regulation of Ca\(^{2+}\) and phosphorus homeostasis and bone remodeling. However, 1,25(OH\(_2\))D\(_3\)/VDR signaling is also involved in immunity regulation, detoxification of xenobiotics, antimicrobial defense, anti-inflammatory and anticancer effects, and cardiovascular protection (420). VDR also activates the mammalian hair cycle, exemplifying an additional VDR function that promotes healthy aging (174). In addition to these multiple actions, the protective vitamin D effects involve a suppressive action on RAS (see The KLOTHO-Vitamin D-RAS Connection and Fig. 4).

However, renal capacity to transform 25(OH)D\(_3\) to the active 1,25(OH\(_2\))D\(_3\) decreases with age, and CYP24A1 [the enzyme responsible for 1,25(OH\(_2\))D\(_3\) degradation] gene expression increases with aging and is accompanied by increased 1,25(OH\(_2\))D\(_3\) clearance (13, 275, 407). These observations suggest that loss of vitamin D actions may contribute to age-related disease (79). In this context, vitamin D deficiency is highly prevalent among the elderly (125).

**KLOTHO-mTOR and KLOTHO-Sirtuin connections.** KLOTHO levels are reduced in aging subjects and in the kidneys of diabetic nephropathy patients. A relation between KLOTHO and mTOR was recently revealed in a study showing that in male klotho\(^{-/-}\) mice with streptozotocin-induced diabetes, KLOTHO deficiency aggravated early diabetic nephropathy by upregulating both renal mTOR and TGF-B1/mTOR signaling (255).

Regarding KLOTHO as a tumor suppressor, in cultured gastric cancer cells KLOTHO downregulates type 1 IGF receptor (IGF1R) phosphorylation and the ensuing activation of IRS-1/PI3K/AKT/mTOR signaling, thereby modulating cell proliferation, apoptosis, and autophagy (439). In human embryonic kidney cells, the KLOTHO-FGF19 complex attenuated the activation of AKT and mTOR accompanied by ERK1/2 activation, which is thought to underlie pro-apoptotic actions (266). Also, in klotho\(^{-/-}\) mice, KLOTHO deficiency promoted vascular dysfunction and hypertension that were mediated by mTOR upregulation (380).

Regarding the relation between KLOTHO and sirtuins, resveratrol induced SIRT1 mRNA expression in mouse kidney, and this was involved in regulating KLOTHO mRNA expression (182).

**The Renin-Angiotensin System**

Originally the renin-angiotensin system (RAS) was recognized as a group of extracellular enzymes [renin, angiotensin-converting enzyme (ACE)] and circulating peptides [angiotensinogen, angiotensin I, angiotensin II (ANG II), and related peptides] exclusively involved in the regulation of systemic blood pressure and renal electrolyte balance through its effects on vascular tone, aldosterone release, kidney sodium absorption, water consumption, sympathetic activity, and vasopressin secretion, i.e., the circulating RAS. Later came the discovery of the local or tissue RAS that comprises components produced in the heart, kidney, brain, vasculature, adipose tissue, adrenal...
gland, and immune system, serving as both autocrine regulators of organ functions and contributors to cardiovascular homeostasis (313) that can act independently or closely connected with the circulating RAS.

Present knowledge indicates that the RAS consists of two antagonistic branches: 1) a pressor/proliferative/fibrotic branch composed of ACE, ANG II (a product of ACE acting on ANG I), and the ANG II type 1 receptor (AT1R) that is referred to as the ACE-ANG II-AT1R axis and 2) a counterbalancing branch, or Ang-(1–7)-angiotensin-converting enzyme 2 (ACE2)-Mas axis, that comprises ACE2, angiotensin (1–7) [Ang-(1–7); generated by hydrolysis of ANG II by ACE2], and the Mas receptor that mediates the vasodilator, antiproliferative, antifibrotic, and antithrombotic actions of Ang-(1–7) (133). The existence of these two branches gives birth to the hypothesis that downregulation of Ang-(1–7) activity or expression increases the vulnerability of the cardiovascular system to the deleterious effects of ANG II (133).

**ANG II-initiated signaling through G proteins and arrestins.**

The numerous and varied actions of ANG II are triggered via highly complex and specific signaling pathways that are induced after ANG II’s binding to distinct receptors. The majority of the physiological and pathophysiological effects of ANG II are mediated by its AT1R and type 2 receptors (AT2R) (177, 384). When activated, the ubiquitous AT1R that is predominantly expressed in cardiovascular cells promotes vasoconstriction and renal tubular sodium and water retention (40), inflammation and tissue fibrosis (403), and tissue-damaging immune responses (reviewed in Ref. 384) and contributes to the aging process (26), whereas activation of the AT2R exerts opposing AT1R effects (reviewed in Ref. 384). RAS receptors were originally thought to reside only in tissues directly involved in the regulation of blood pressure and electrolyte balance; however, at present RAS receptors have been described in almost every tissue (132). The AT1R belongs to a family of seven transmembrane receptors, also known as guanine nucleotide-binding protein (G protein)-coupled receptors, which were traditionally thought to mediate downstream signaling exclusively through heterotrimeric G proteins (177).

ANG II signals conveyed by G proteins in the AT1R activate phospholipase C that, followed by stimulation of Ca2+ signaling, participates in vasoconstriction and cardiac hypertrophy, whereas activation of phospholipase A2 is involved in ANG II-induced growth of vascular smooth muscle cells and cardiac hypertrophy (reviewed in Refs. 177 and 278). G proteins also participate in ANG II-induced phosphorylation and activation of extracellular signal-related kinases (ERKs), c-Jun NH2-terminal kinases (JNKs), and p38 (214), all of which belong to the family of MAPK, a group of kinases that act successively and serve as central regulators of cellular growth and differentiation. MAPK activation by ANG II is involved in the promotion of cardiac hypertrophy and vascular smooth muscle cell growth (215, 429).

However, G protein-coupled receptors, including the AT1R, can also signal through a G protein-independent pathway that involves the participation of the scaffolding proteins β-arrestin1 and β-arrestin2 (212, 430). Although β-arrestin1 and -2 were considered to serve only as the main mediators of G protein-coupled receptor desensitization and internalization (419), they were later found to function as signal transducers that activate a broad set of intracellular signaling molecules (110, 245).

Furthermore, the G protein and β-arrestin pathways give rise to different biological responses, and can be modulated independently with “biased ligands” (431); whereas standard agonists/antagonists can activate/inactivate the totality of a receptor’s signaling network, biased ligands selectively affect some signals but evade, or even inactivate, others deriving from the same receptor.

The signaling responses induced by ANG II binding to the AT1R and delivered through arrestins to mTORC1 are described in The mTOR-RAS Connection and shown in Fig. 1. **ANG II and oxidants.** At present, it is clear that both the increased generation of cellular ROS and activation of redox-sensitive signaling cascades are critical events involved in ANG II actions (14, 405). After binding to its AT1Rs, ANG II triggers intracellular superoxide (O2−) production by activating NAD(P)H oxidase (157, 218, 278) and uncoupling endothelial nitric oxide synthase (NOS) (288).

ANG II also enhances nitric oxide (NO) generation (321) and, since the reaction of NO with O2− generates peroxynitrite, it can promote the production of both ROS and RNS and reduce NO availability (288, 402). Under normal physiological conditions, ANG II-mediated ROS and RNS production, and the resulting stimulation of redox-sensitive signaling pathways, is closely regulated (402). However, under conditions associated with RAS overactivation, such as hypertension, diabetes (338, 404), and normal aging (23, 159, 398, 425), ANG II-dependent oxidant generation becomes a significant contributor to cell oxidation and tissue damage (102, 311).

In response to ANG II-mediated ROS production, several growth pathways are activated (350). ANG II binding to AT1R leads to the redox-sensitive transactivation of endothelial growth factor receptor (413) and platelet-derived growth factor receptor (349). ANG II also transactivates the insulin-like growth factor (IGF) receptor (IGFR), at least in part by a redox-sensitive mechanism (119).

In endothelial cells, ANG II triggers a signaling cascade that involves ROS and p38 MAPK activation and induces intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 (VCAM-1) expression by activating NF-κB (87).

In addition, ANG II promotes the ROS-dependent activation of c-Src tyrosine kinase, which plays a pivotal role in regulating the genetic expression of extracellular matrix proteins and cell adhesion molecules, and is involved in ANG II-mediated cytoskeletal reorganization (188).

In this setting, antioxidants and antioxidant enzymes inhibit the regulatory effects of ANG II on Ras/Raf/ERK and activator protein-1 (AP-1) signaling pathways (323, 373). In vascular smooth muscle cells from spontaneously hypertensive rats (SHR), ANG II was shown to enhance the activation of NF-κB and AP-1, two transcription factors modulated by the cellular redox status (345).

**ANG II and mitochondrial function.** In addition to the sources of ANG II-mediated ROS production mentioned above, this peptide was found to stimulate mtROS production. In rat endothelial cells, ANG II–induced mtROS activate redox-sensitive NF-κB, followed by stimulation of VCAM-1 expression, a cytokine involved in atherosclerosis lesion formation (322). In mice, acute (24-h) and chronic (14-day) ANG
II infusion led to decreased cardiac expression of mitochondrial electron transport chain and TCA cycle genes (238). In the short-term treatment, ANG II also promoted the depression of mitochondrial metabolism and MnSOD gene expression, and enhanced the expression of genes for proteins that protect against oxidative stress, such as thioredoxin, glutaredoxin, and transferrin receptor 1 (238). This evidence supports previous observations indicating a role for ANG II in the depression of mitochondrial energy metabolism (62, 352, 381). Moreover, in rat vascular smooth muscle cells and aorta in vivo ANG II lowers mitochondrial membrane potential as a result of stimulation of mtROS production (217). Concerning cell signaling modulation by mitochondria, JNK is both a downstream target of AT1R-dependent signaling (225) and a regulator of AP-1 activity. Because AP-1 regulates cytochrome c expression (437), it was suggested that JNK may facilitate changes in the mitochondrial content of cytochrome c in response to ANG II (241).

Another link between ANG II and mitochondrial dysfunction is suggested by data showing that mitochondrial p66Shc plays a crucial role in ANG II-induced myocardial remodeling (154). p66Shc, a protein partially localized in the mitochondrial intermembrane space, was suggested to contribute to mtROS production by subtracting electrons from cytochrome c, and transferring them to oxygen to generate superoxide.

ANG II administration was found to increase protein oxidation, mitochondrial DNA (mtDNA) content, and deletion frequency in mouse cardiac mitochondria, all of which were attenuated in MCAT mice (that overexpress human catalase in mitochondria) (91); in addition, ANG II-induced cardiac hypertrophy was mitigated in MCAT mice. These observations point to a causal role of mtROS in ANG II-induced mitochondrial dysfunction and cardiomyopathy. Notably, mitochondrial dysfunction was associated with endothehial dysfunction as indicated by the decrease of endothelial NO production (115). The molecular mechanisms involved in ANG II-mediated mitochondrial dysfunction include protein kinase C activation, which in turn activates bovine aortic endothelial cell NAD(P)H oxidase and stimulates peroxynitrite formation (115).

Zhang et al. (450) reviewed evidence supporting the notion that, by activating NAD(P)H oxidase, ANG II induces the generation of ROS, that in turn activate downstream mitochondrial ATP-dependent potassium channels leading to a burst of mtROS, which subsequently activate downstream signaling cascades that include the JNK and p38 MAPK, thereby promoting cell apoptosis, differentiation, and hypertrophy.

The involvement of mtROS in cardiac signaling activated by RAS components and the participation of mtROS in physiological and pathological responses were recently revised by De Giusti et al. (106). Of note, ANG II promotes the release of endothelin-1 in cardiomyocytes (314), and both ANG II and endothelin-1 (107) can activate NAD(P)H oxidase and the consequent mtROS production.

Other evidence indicates a direct interaction between ANG II and mitochondrial components. 125I-labeled ANG II was detected in heart, brain, and smooth muscle cell mitochondria and nuclei (341, 378). In rat adrenal zona glomerulosa, renin, angiotensinogen, and ACE were detected within intramitochondrial dense bodies (315), and in rat cerebellar cortex mitochondria ANG II immunoreactivity was also found (124). More recently, ANG II was found to bind to AT1R and AT2R present in the inner mitochondrial membrane of both human and mouse cells; also, activation of the mitochondrial ANG II system was associated with mitochondrial NO generation and the modulation of mitochondrial respiration (1). Interestingly, mouse aging was associated with increased mitochondrial AT1R and reduced mitochondrial AT2R density in kidney tubular cells, and these effects were abolished by chronic AT1R blocker (ARB) (losartan) treatment (1).

As a result of the above, it is apparent that the effects of ANG II on mitochondria may be either 1) dependent on NAD(P)H oxidase activation or 2) direct.

In support of the proposed direct actions of ANG II, in rat embryonic vascular smooth muscle cells ANG II can exert effects from the cell interior, independent from extracellular actions, and seemingly through binding to intracellular receptors different from AT1R and AT2R (134), or to AT1 nuclear receptors (254). Internalization of ANG II can be mediated by internalization of AT1R bound to ANG II, as was reported in rat aortic smooth muscle cells (9), hepatocytes (195), human vascular smooth muscle cells (33), and renal proximal tubule epithelial cells (396). In some cells, ANG II internalization is mediated by megalin, although this mechanism is thought to mark internalized ANG II for decomposition (153).

In relation to the consequences of ANG II acting on mitochondria, we have shown that, in rodent models of hypertension, diabetes, and aging, ANG II blockade not only attenuates oxidant production but also improves mitochondrial function (98, 103, 104). In SHR, losartan treatment prevented the alterations in renal mitochondrial H2O2 production rate, mitochondrial membrane potential, uncoupling protein-2 (UCP-2) content, and MnSOD, mitochondrial NOS, and cytochrome oxidase activities that occurred in untreated SHR (104, 105). UCPs can modify mitochondrial energy output, and modulate mitochondrial oxidant production, by uncoupling mitochondrial electron transport from ATP production.

In streptozotocin-diabetic rats, losartan protected kidney mitochondria against changes in H2O2 production rate, membrane potential, and pyruvate content, without reducing plasma glucose levels (98). In both studies, amlodipine (a Ca2+ channel blocker) or losartan similarly reduced blood pressure, but only losartan protected kidney mitochondria.

Also, the modulation of heart and liver mitochondrial NOS activity and H2O2 production by the ACE inhibitor enalapril has been observed in rats (38, 87). Moreover, the expression of genes related to fatty acid β-oxidation, mitochondrial proton-electron coupling, and oxidative phosphorylation was upregulated in captopril-treated diabetic animals, suggesting that RAS inhibition with ACE inhibitors may protect the myocardium by enhancing energy supply (75).

**RAS-bl and Aging**

Early work showing that in hypertension, heart failure, and chronic renal failure, RAS-bl with ACE inhibitors and later with ARB affords cardiac and renal benefits not limited to their antihypertensive effects (reviewed in Ref. 205) pointed to ANG II acting as a growth (148) and profibrotic factor (41). Later, work from our laboratory showed that chronically administered enalapril attenuates age-related renal (129) and myocardial changes (130), increases mitochondrial number in heart and liver cells, prevents age-associated weight loss, and
increases survival in aging mice (130). Based on these observations, we proposed that enalapril treatment altered the natural aging mechanisms and that RAS played a role in aging, and hypothesized that enalapril acted as an antioxidant and prevented mitochondrial injury. We also found that RAS-bl with either an ACE inhibitor (enalapril) or an ARB (losartan) increases survival in rats (20). A study led by others showed that AT_{1}R gene disruption also promotes longevity in mice, and protects against cardiac and vascular damage and oxidative damage in multiple organs, and increases mitochondrial number and the expression of two prosurvival genes, nicotinamide phosphoribosyl transferase and SIRT3, in the kidney compared with wild-type mice (26).

Next we focused our research on RAS-bl actions on mitochondrial function by studying its effects in hypertension (104) and diabetes models (98), two conditions known to independently involve mitochondrial dysfunction and RAS derangements (327, 365). The beneficial mitochondrial actions of ACE inhibitors and ARB were described in ANG II and mitochondrial function.

Other reports showed that ANG II inhibition improved cardiac mitochondria energy production (289, 290, 307), and in diabetic rats captopril treatment upregulated the expression of genes related to energy production (74). Among the potential factor(s) that may mediate the effects of ANG II inhibitors on mitochondrial function, mitochondrial NO was suggested to contribute to the renal mitochondrial actions of enalapril (317), and ARBs were found to modulate UCP mRNA levels in mouse brown adipose tissue (12) and rat liver (78), or UCP protein content in rat kidney (103, 317).

In addition, long-term ACE inhibitor or ARB treatments provide other age-related protective actions such as the reduction of body fat mass, and improvements in physical performance (61) and cognitive function (131).

Furthermore, 16.5-mo-long enalapril or losartan administrations were unable to prevent the age-dependent accumulation of rat liver mtDNA “common deletion,” but they both attenuated the decrease of mtDNA content (100), which was accompanied with enhanced NRF-1 and PGC-1α mRNA contents. The above observations suggest an explanation to the enalapril- and losartan-mediated improvement in mitochondrial function and lowering of oxidant production (100), since mitochondrial respiratory capacity is positively related to both the absolute number of mtDNA molecules (28) and NRF-1 and PGC-1α transcription levels (135); also, PGC-1α prevents from damage inflicted by increased ROS production (382).

When analyzing RAS-bl effects on aging, it is necessary to consider that, apart from its hemodynamic effects, ANG II is a pleiotropic peptide that, by acting on AT_{1}R and AT_{2}R inhibitors, prevents from damage inflicted by increased ROS production (382).

The mTOR-RAS Connection

Early work showed that, in cultured rat cardiomyocytes, ANG II-induced hypertrophy is accompanied by decreases in both AMPK activation and mTOR inhibition, and preincubation with an AMPK activator prevents mTOR activation and decreases protein synthesis as well as the expression of hypertrophy markers (389). Later, the AT_{1}R was found to mediate the induction of cardiomyocyte hypertrophy by tri-iodothyronine through the activation of AKT/blycogen synthase kinase-3β (GSK-3β)/mTOR signaling. AT_{1}R blockade (with losartan) and AT_{1}R silencing (with a small-interfering RNA) completely blocked cardiomyocyte hypertrophy, and ameliorated or totally blocked AKT/GSK-3β/mTOR activation (112). In transgenic rats that overexpress the mouse renin (Ren-2) gene, the development of tubulointerstitial fibrosis was dependent on ANG II-mediated activation of mTOR/S6K1 signaling, and AT_{1}R-blockade (telmisartan) reduced kidney fibrosis and mTOR/S6K1 activation (433); also, aldosterone, through its mineralocorticoid receptor, promoted proximal tubule injury by increasing mTOR/phosphorylated S6K1 signaling, and treatment with spironolactone (a mineralocorticoid receptor antagonist) mitigated tissue damage and reduced mTOR/S6K1 phosphorylation (434). In mice, 3

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In rat cardiomyocytes, ANG II treatment upregulates AT_{1}R levels, and induces mitochondrial dysfunction and cell hypertrophy, whereas RAS-bl (losartan) reduces ANG II-induced AT_{1}R upregulation, prevents mitochondrial impairment, and stimulates AMPK activity through a yet unraveled mechanism (176). Also, metformin, a potent AMPK activator, prevented AT_{1}R upregulation. These results suggest a negative reciprocal relation between AMPK and AT_{1}R and are consistent with previous observations showing that the ARBs telmisartan and candesartan stimulate AMPK activity in cultured myotubes (128) and rat hypothalami (269).

The Sirtuin-RAS Connection

As already mentioned above, AT_{1}R gene disruption promotes longevity in mice, in association with protection against oxidative damage in multiple organs, cardiac and vascular damage, increased numbers of mitochondria, and enhanced expression of two prosurvival genes (nicotinamide phosphoribosyl transferase and SIRT3) in the kidney compared with wild-type mice (26). The relation between RAS and sirtuins was confirmed by later work showing that telmisartan, an ARB that functions as a partial agonist of PPAR-γ, improved insulin sensitivity in obese db/db mice fed a high-fat diet through a PPAR-γ-independent pathway, and at least in part by upregulating the skeletal muscle AMPK/SIRT1 pathway (375). Also, in ANG II-treated mice, cardiac insulin resistance and hypertrophy were mediated by ANG II-induced reduction in SIRT3 levels (291), the loss of skeletal muscle force by increases in NAD(P)H oxidase expression, and the subsequent ROS-induced downregulation of IGF-1, PGC-1α, and SIRT1 (199). In ANG II-treated human umbilical vein endothelial cells, the ACE inhibitor zofenoprilat inhibited superoxide production, cell apoptosis, and NF-kB activation and reverted SIRT1 expression (375). Also, metformin, a potent AMPK activator, prevented AT_{1}R upregulation. These results suggest a negative reciprocal relation between AMPK and AT_{1}R and are consistent with previous observations showing that the ARBs telmisartan and candesartan stimulate AMPK activity in cultured myotubes (128) and rat hypothalami (269).

PPARs and RAS-bl

PPARs are nuclear transcription factors that regulate the expression of a number of genes related to lipid metabolism and energy homeostasis (29, 76, 421). Activation of PPAR-α results in the increased expression of many nuclear genes associated with mitochondrial function, including those involved in fatty acid β-oxidation (361, 362) and mitochondrial proton leak (210, 299) and those encoding antioxidant enzymes, i.e., MnSOD and catalase (393). PPAR-γ, which is highly expressed in adipocytes, is involved in adipocyte differentiation and controls the expression of lipid storage genes. Another relevant function of PPAR-γ is the promotion of
inhibits 1 (reviewed in Ref. 97). In turn, the increased FGF23 content with FGF23 signaling and results in elevated FGF23 levels KLOTHO expression (286, 348, 443, 453), which interferes VDR have not been found; however, ANG II reduces renal
is regulated by the RAS, the issue is not completely settled.

Although abundant data suggest that vitamin D metabolism is regulated by the RAS, the issue is not completely settled. Direct effect(s) of RAS components on 1α-hydroxylase or VDR have not been found; however, ANG II reduces renal KLOTHO expression (286, 348, 443, 453), which interferes with FGF23 signaling and results in elevated FGF23 levels (reviewed in Ref. 97). In turn, the increased FGF23 content inhibits 1α-hydroxylase, leading to the lowering of 1,25(OH)2D3 production (97). Of note, in chronic kidney disease, high FGF23 levels or FGF23 resistance induced by insufficient KLOTHO availability are associated with dysfunctional endo-
thelia, cardiovascular morbidity and mortality, and disease progression (435). In this scenario, it was proposed that chronic kidney disease, a highly prevalent condition characterized by noxious derangements of the internal environment that stimulate cell senescence and early age-related changes, be consid-
ered a clinical model of premature aging (385).

The above relations between KLOTHO, vitamin D, and RAS are summarized in Fig. 4.

In support of RAS/soluble KLOTHO connections, experi-
mental in vitro and in vivo studies showed that RAS-bl in-
teracts with KLOTHO and protects against cell senescence and/or apoptosis (20). Furthermore, in type 2 diabetic patients with diabetic kidney disease, treatment with the ARB valsartan was associated with increases in soluble KLOTHO, accompanied with reduced serum phosphate and cardio renal improvements (207).

Further evidence for RAS/VDR signaling interactions was provided by our study in rats subjected to obstructive nephropathy (a model of RAS upregulation) and treated with the VDR activator paricalcitol, where VDR activation attenuated renal fibrosis, apoptosis, and mitochondrial injury, accompanied by reduction of mitochondrial AT1R mRNA contents and VDR upregulation (147). In SHR, the same renal protective effects were brought about by 4 mg paricalcitol, enalapril, or combined paricalcitol-enalapril treatments. Importantly, both paricalcitol and enalapril improved VDR expression (146).

\section*{Calorie Restriction and Aging}

CR, without malnutrition, is one of the most consistent interventions to reduce the rate of aging in an ample range of species, from yeast to humans (140). In vertebrates, including rodents and nonhuman primates, CR increases mean and maximum lifespan and retards both the decay of physiological functions and the appearance of diseases associated with aging, such as hypertension, diabetes, nephropathy, cardiovascular disease, and cancer (66, 94, 264, 276, 383). However, the molecular foundation underlying the consequences of CR on aging continues to be only partially resolved. The reduction of metabolic rate represents one explanation for the beneficial effects of CR observed in different animal species. This reduction leads to lower O2 consumption, ROS generation (2, 126), and oxidatively damaged proteins (141, 244, 448), lipids (230, 243, 309), and DNA (156, 263) than do ad libitum-fed rodents and primates. With regard to its effects on mitochondria, long-term CR lowers the rate of mitochondrial H2O2 produc-
tion and decreases the levels of mtDNA oxidative damage in rat liver, heart, skeletal muscle, and brain (117, 156, 263, 355). In addition, CR was shown to increase the expression of UCP2 in mice and in humans (283, 438), which may explain the effects of dietary manipulation on the reduction of mito-
chondrial H2O2 production. Analogous antioxidant and protective effects were observed in different animal species as well as in different tissues and cells (31). Also, KLOTHO protein and mRNA expression were induced by CR in the kidney of aging male rats (287).

\section*{PPARs and CR}

In rodents, PPARs seem to play an impor-
tant role in the delay of aging caused by CR (86, 312). In the rat, PPAR nuclear protein, mRNA level, and DNA-binding activity were shown to decrease with age, whereas CR blunted these reductions (391). Alternatively, using microarray tech-
nology, PPAR target genes were upregulated early and in-
tensely in the livers of CR mice (22). Also, both in vitro and in vivo (rat liver) CR were shown to induce PGC-1α-mediated mitochondrial biogenesis while maintaining bioenergetic effi-
ciency and lowering oxidative stress (262).

Although abundant studies have repeatedly shown that CR attenuates mtROS production (reviewed in Ref. 155), a recent
review of rodent literature showed that the effects of CR on oxidative stress are complex and seem to be dependent on multiple influences, including the duration of the intervention, the species, tissue, and sex studied, and the nature of the antioxidants and ROS examined (422). It should also be considered that the term CR has been used to refer to various diets. Among these, the most frequently used are intermittent fasting, food restriction, and food restriction with micronutrient supplementation, each of which displays specific effects and mechanisms of action (67). In this scenario, Sanz et al. pointed out that lipid or carbohydrate restriction has no effect on mtROS production, whereas protein restriction reduces mtROS generation in rat liver independent of energy intake (356). In addition, methionine restriction, which increases longevity in rodents (280, 337), reduces mtROS production and decreases oxidative damage in rat heart and liver mitochondria, suggesting that decreased methionine ingestion may contribute to the antiaging effects of CR (356).

The role of sirtuins in CR. Although the idea that sirtuins might be involved in the antiaging effects of CR has been challenged by contradicting findings, a recent analysis (160) of newer evidence robustly encourages the notion that sirtuins mediate longevity from yeast to rodents; as to humans, two gene variants (single nucleotide polymorphisms) of the SIRT3 gene were associated with longevity (6).

In mice, SIRT3 induction by CR mediates favorable antiaging actions by increasing the mitochondrial reduced-to-oxidized glutathione ratio (379). In mice, SIRT6 deficiency promotes age-related anomalies (296), and CR increases SIRT6 levels in rats (204), pointing to the role of SIRT6 in aging. Recent work showed that 40% CR for 6 mo upregulates mouse liver mRNA levels of all seven sirtuins (138).

Furthermore, 7 wk CR in overweight patients upregulated phosphorylated SIRT1 in peripheral blood mononuclear cells; also, serum collected from CR rats induced SIRT1 expression and cell survival in HEK 293T cells (83), and/or serum collected after CR in humans induced SIRT1 expression and cell survival in HEK 293T cells (8). Summarizing, in rodents, SIRT1 to SIRT7 are upregulated in peripheral blood mononuclear cells; also, serum collected from CR rats induced SIRT1 expression and cell survival in HEK 293T cells (83), and/or serum collected after CR in humans induced SIRT1 activity and mitochondrial biogenesis in cultured human hepatoma cells (8).

AMPK activation also includes increasing the number of mitochondria, and using mitochondrial substrates to generate energy (169, 201). To attain the latter purpose, AMPK-mediated modulation of the activity of transcription factors (e.g., myocyte enhancer factor-2, PPARα, and PPARδ) and coactivators, such as PGC-1α (comprehensively reviewed in Ref. 55), regulates the expression of genes involved in mitochondrial biogenesis, energy production, and protection against ROS. Thus, AMPK functions as a key regulator of mitochondrial biogenesis and metabolism, which suggests that this enzyme may both impact on lifespan and health span (351) and play a role in the favorable actions of CR (56).

CR, mTOR, and AMPK. In yeast lacking one of the two yeast TOR genes, CR did not further extend lifespan (200), and in a C. elegans genetic model of CR lifespan was not prolonged when TOR was inhibited with a double-stranded RNA (167), suggesting that CR and TOR inhibition enhance lifespan through the same mechanism. Contrarily, rapamycin marginally extended lifespan in flies subjected to CR (32), and inhibition of S6K, one of the main TOR targets, extended lifespan in a C. elegans model of CR (168), indicating that although CR and TOR inhibition increase lifespan through overlapping mechanisms, they also affect some noncommon pathways.

Similarly, the fact that both CR and rapamycin can extend rodent lifespan suggested that they operate by analogous mechanisms. A study aimed at testing this notion in mice (138) showed that both CR and rapamycin reduce mTOR signaling (as assessed by ribosomal protein S6 phosphorylation) and correspondingly increase autophagy; however, only CR decreased the fat mass, and increased insulin sensitivity, SIRT1-7 gene expression, and the reduced-to-oxidized glutathione ratio. In addition, rapamycin-mediated lifespan extension in genetically heterogeneous mice was not accompanied by some metabolic and endocrine changes that are present in mice subjected to CR, and the hepatic expression of xenobiotic metabolism genes was different for each of these interventions (282). These results suggest that, although CR and rapamycin might extend lifespan through (a) shared pathway(s), other(s) are differently affected.

As a fuel-sensing enzyme, AMPK is activated by diminished cell energy status to reestablish energy equilibrium by triggering both a short- and a long-time response. The short-time response consists of upregulating catabolic reactions that generate ATP, such as fatty acid oxidation, and suppressing anabolic reactions that use up ATP but are not seriously required for survival, such as protein and triglyceride synthesis, cell growth, and division. The immediate whole body effects of AMPK activation also include augmenting glucose uptake by the skeletal muscle (229) and reducing the rates of glycolysis (60). The long-time response initiated after AMPK activation includes increasing the number of mitochondria, and using mitochondrial substrates to generate energy (169, 201). To attain the latter purpose, AMPK-mediated modulation of the activity of transcription factors (e.g., myocyte enhancer factor-2, PPARα, and PPARδ) and coactivators, such as PGC-1α, needs to be deacetylated by SIRT1 (342). Furthermore, AMPK activation was shown to induce SIRT1 function, and how this may affect aging was recently reviewed (344). Interestingly, the capacity of AMPK to respond to upstream signals is markedly diminished in aged rodent tissues (reviewed in Ref. 351).

In mouse skeletal muscle, AMPK regulates mitochondrial biogenesis by directly phosphorylating PGC-1α (192, 246), which enables PGC-1α deacetylation by SIRT1 (58). Of note, its acetylation status firmly controls PGC-1α’s activity (194). Under basal conditions, PGC-1α is intensely acetylated and therefore moderately active (247); to acquire maximal activity, PGC-1α needs to be deacetylated by SIRT1 (342). Furthermore, AMPK activation was shown to induce SIRT1 function, and how this may affect aging was recently reviewed (344). Interestingly, the capacity of AMPK to respond to upstream signals is markedly diminished in aged rodent tissues (reviewed in Ref. 351).

In this scenario, activation of AMPK is the most studied stimulus for mTOR suppression (see mTOR, Sirtuins, and KLOTHO: The Most Consistently Altered Targets in Aging) and the ensuing inhibition of anabolic processes (161, 187). Because in various organisms mTOR downregulation extends lifespan in a CR-like manner, the AMPK-mediated inhibition of mTOR affords a potential mechanism for AMPK to impact on lifespan (56). However, evidence exists against AMPK acting as an agent in CR actions, and the issue of AMPK being activated by CR is mostly unsettled (reviewed in Ref. 56).

The GH/IGF pathway and CR. GH is released into the circulation by the anterior pituitary and, after binding its GH...
receptors in various tissues, it regulates overall body growth and also influences metabolism, immunity, heart function, cognitive function, and aging (reviewed in Ref. 198) by triggering a variety of signaling cascades that lead to cytoskeletal reorganization, cell proliferation, differentiation and migration, apoptosis inhibition, and metabolic pathway regulation (235).

Activation of the GH receptor promotes the synthesis and secretion of IGF-I, mostly from the liver; GH-mediated regulation of IGF-I release from other tissues influences IGF-I’s paracrine or local activities (reviewed in Refs. 49 and 248). IGF-I stimulates DNA, RNA, and protein synthesis, and acts as a mitogen. Notwithstanding that IGF-I serves as GH’s anabolic mediator, GH also acts directly on specific tissues and/or synergizes with IGF-I actions.

In this scenario, GH secretion, and hence that of IGF-I, drops in the course of time in such a way that low circulating levels are present in subjects older than 60 years old (198, 372, 417). Contrarily, reduced GH/IGF-I signaling increases longevity across species, from yeast to mice (140). In humans, evidence from the only available model to evaluate the effects of reduced GH/IGF-I signaling on aging, i.e., Laron Syndrome, an inherited condition caused by mutations that inactivate the GH receptor resulting in IGF-I deficiency (240), showed that lifelong IGF-I insufficiency allows to attain eldersness, even in the presence of pronounced obesity, hyperlipidemia, and a propensity to progress to diabetes and its complications (239). Recent human studies revealed that survival is significantly improved in nonagenarian females, but not males, with IGF-I levels that are lower than the median value, relative to females whose levels exceed the median (279). In the same study, low IGF-I levels foretold enhanced survival in both females and males with a history of cancer.

Of note, CR typically reduces circulating IGF-I levels, and therefore downregulates the GH-IGF-I axis (208). Correspondingly, in GH receptor knockout mice, chronic CR was unable to further improve longevity (35, 36). In GH-resistant or GH-deficient mice, mTOR signaling is attenuated in particular cells and tissues, which suggests that both CR and the GH-IGF-I axis might affect the aging process by interfering with mTOR signaling (36, 424). However, work with long-lived mutant mice showed that disruption of the GH axis and CR share some, but not all, signaling pathways (5, 390).

Finally, IGF-I can rescue many age-related changes in rats, and this protective action is related to mitochondrial function protection (320).

In Fig. 5 we have summarized the most studied changes associated with aging, i.e., alterations in mTOR, Klotho, RAS, sirtuins, PPARs, and AMPK, and their relation to mitochondrial dysfunction.

RAS and IGF Signaling

Regarding the relation between the RAS and IGF signaling, early work showed that ANG II induces vascular smooth muscle cell mitogenesis by increasing the number of IGFR and upregulating IGFR1 mRNA (109, 119). Later, stimulation of the cardiac and systemic IGF-I pathways was found to make a chief contribution to ANG II-induced cardiac remodeling (42). In cultured neonatal rat cardiac fibroblasts, ANG II was shown to increase IGFR1 expression; also, the mitogenic effect of IGF-I was partly mediated by RAS activation and the ensuing upregulation of IGFR1 expression, which was abolished by ACE inhibitors (moexiprilat or enalaprilat) and ARB (CV11974, the bioactive candesartan metabolite) (415). In a rat model of cardiac hypertrophy by aortocaval shunt, IGF-I binding to its receptor in the myofibers was elevated, and treatment with an ACE inhibitor (captopril) or an ARB (losartan) resulted in regression of cardiac hypertrophy accompanied by an attenuation or normalization of increased IGF-I affinity (162). In kidney transplant patients with posttransplant erythrocytosis, treatment with ACE inhibitors reduced both the hematocrit and serum IGF-I; hematocrit and circulating IGF-I levels were positively related, suggesting that IGF-I contributes to the ACE inhibitor-induced decrease of hematocrit in these patients (295).

Paradoxically, ANG II induces skeletal muscle atrophy and proteolysis, and downregulation of IGF-I signaling seems to play a key role in this wasting effect (445). In aged mice, immobilization-induced muscle atrophy was reduced by losartan treatment, and this protective effect was mediated by increased activation of the IGF-I/PI3K/mTOR pathway, the main signaling pathway known to control protein turnover in skeletal muscle (48). However, mRNA and protein expression analysis of the IGF-I/PI3K/mTOR pathway components revealed mild age-associated changes among old sedentary and old active subjects and among young and old mice, suggesting that aging is not associated with downregulation of the IGF-I/PI3K pathway and that sarcopenia (the loss of muscle mass and force) is not the result of proteolytic system upregulation. Furthermore, genetic activation of the IGF-I/PI3K pathway in old mice showed that sarcopenia is
not delayed, but accelerated, when protein degradation pathways are impaired (353).

The above contrasting evidence might be explained by the complexity of the IGF system, which is composed of two ligands, IGF-I and IGF-II, that can bind two receptors, IGF1R and type 2 IGF receptor, and at least six high-affinity IGF-binding proteins that modulate IGF-I and IGF-II bioavailability and can potentiate IGF effects (137, 377). In addition, the IGF system not only can cross talk with the insulin receptor but can signal through hybrid insulin/IGF receptors, formed by heterodimerization, that are thought to improve cell responses to IGFs (377). In this context, a recent study showed that the proliferative response of cultured immortalized ovarian cancer cells is dependent on a quantitative equilibrium between the different components of the IGF system (399).

**CR and RAS-bl**

In a previous review (101) we summarized data showing that, apart from retarding age-related alterations, CR and RAS-bl display a number of converging effects in rodents, primates, and humans, i.e., they delay the manifestations of hypertension, diabetes, nephropathy, cardiovascular disease, and cancer; increase body temperature; reduce body weight, plasma glucose, insulin, and IGF-I; ameliorate insulin sensitivity; lower protein, lipid, and DNA oxidation, and mitochondria...
drial H₂O₂ production; and increase UCP-2. Interestingly, many of these overlapping effects involve changes in mitochondrial function. We hypothesized that PPAR modulation by RAS-bl participated in the protection of mitochondria during rodent aging, parallelling an effect already reported for CR. Among the potential mechanisms underlying RAS-bl’s mitochondrial benefits, we included TGF-β downregulation and KLOTHO upregulation. While reinforcing the role of KLOTHO upregulation, the present actualization of this topic has expanded the converging effect of CR and RAS-bl to include mTOR downregulation; upregulation of sirtuin, KLOTHO, and VDR expression; and stimulation of AMPK activity (Fig. 6). The proposed involvement of mTOR, VDR, and AMPK in the age-retarding effects of RAS-bl was drawn from evidence obtained mainly in nonaging models in rodents, with some data from nonhuman primates, and supported by indirect evidence in humans. However, many aspects of this proposal require further study.

Concluding, the available evidence endorses the idea that RAS-bl is among the interventions that may turn out to provide relief to the spreading issue of age-associated chronic disease.

It is worth mentioning that, although rapamycin has a robust experimental backing as a potential intervention to retard aging in mammals, it has a variety of unwanted side effects that suggest that rapamycin might turn out to be unacceptable for human use. On the other hand, ACE inhibitors and ARB are widely used human pharmaceutical agents; therefore, if further research reveals that RAS-bl is beneficial for human longevity and age-related diseases, abundant data will be already available related to their safety.

Finally, although RAS-bl and CR share several downstream signals, the issue of whether both interventions act in separate pathways or converge in a joint pathway to retard aging awaits definitive tests. One way to actually prove this point would be to assess whether RAS-bl can further extend lifespan/retard the signs of aging in animals subjected to CR.

**Summary**

Considering that 1) mTOR inhibition extends lifespan, 2) KLOTHO functions as an aging-suppressor gene, 3) sirtuins mediate longevity, 4) loss of vitamin D actions may contribute to age-related disease, 5) AMPK functions as a key regulator of mitochondrial biogenesis and metabolism, suggesting that this enzyme may both impact on lifespan and health span, 6) reduced GH/IGF-I signaling increases longevity across species, from yeast to mice, and 7) RAS-bl downregulates mTOR and GH/IGF-I signaling, upregulates KLOTHO, sirtuin, and VDR expression, and stimulates AMPK activity, it is feasible that at least some of the benefits of RAS-bl in aging are mediated through the modulation of mTOR, klotho, and sirtuin expression and vitamin D, AMPK, and IGF-I signaling. Figure 6 summarizes the relation between mTOR, sirtuins, KLOTHO, PPARs, AMPK, and mitochondrial function and their potential involvement in the beneficial actions of RAS-bl and CR in retarding the aging process.

In light of these findings, we can answer the questions posed in the first paragraph of this review as follows: 1) Do these interventions attenuate aging by interfering with common mechanisms? Yes, CR and RAS-bl downregulate mTOR and IGF-I; upregulate KLOTHO, sirtuins, and VDR expression; and enhance AMPK activity, all of which have beneficial effects on mitochondrial function; 2) Do the mechanisms that are interfered with by CR and RAS-bl mutually affect each other? Yes, there are complex interactions between mTOR, KLOTHO, and sirtuin signaling; 3) Are there any pathways involved exclusively with a certain intervention? Because of the complexity of the RAS, whose components and functions are continuously engrossed by new discoveries, it is evident that the presently proposed connections referring to RAS’s role in the aging process are far from being exhausted; similarly, the impact of CR on multiple metabolic pathways forewarns that science is still a long way from having cleared the whole picture. Although RAS-bl and CR share several downstream signals, one way to actually prove the overlap would be to assess whether RAS-bl can further extend lifespan/retard the signs of aging in animals subjected to CR.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

Author contributions: E.A.M.d.C. and F.I. conception and design of research; E.A.M.d.C. performed experiments; E.A.M.d.C. analyzed data; E.A.M.d.C. interpreted results of experiments; E.A.M.d.C. prepared figures; E.A.M.d.C. drafted manuscript; E.A.M.d.C., F.I., and L.F. edited and revised manuscript; E.A.M.d.C., F.I., and L.F. approved final version of manuscript.

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RAS BLOCKADE RETARDS AGING THROUGH mTOR, KLOTHO, AND SIRTUIN

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RAS BLOCKADE RETARDS AGING THROUGH mTOR, KLOTHO, AND SIRTUIN

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