Review

Atheroprotective effects of antioxidants through inhibition of mitogen-activated protein kinases[1]

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ABSTRACT

Reactive oxygen species (ROS) have been known to play an important role in the pathogenesis of atherosclerosis and several other cardiovascular diseases. It is now apparent that ROS induce endothelial cell damage and vascular smooth muscle cell (VSMC) growth and cardiac remodeling, which are associated with hypertension, atherosclerosis, heart failure, and restenosis. Several lines of evidence have indicated that ROS and mitogen-activated protein (MAP) kinases were involved in vascular remodeling under various pathological conditions. Recently, it was also reported that MAP kinases were sensitive to oxidative stress. MAP kinases play an important role in cell differentiation, growth, apoptosis, and the regulation of a variety of transcription factors and gene expressions. Bioflavonoids and polyphenolic compounds are believed to be beneficial for the prevention and treatment of atherosclerosis and cardiovascular diseases. One of the most widely distributed bioflavonoids, 3,3´,4´,5,7-pentahydroxyflavone (quercetin) and its metabolite quercetin 3-*O*-β-*D*-glucuronide (Q3GA) inhibited Angiotensin II-stimulated JNK activation and resultant hypertrophy of VSMC. Several studies have suggested that various antioxidants including probucol, *N*-acetyl-*L*-cysteine, diphenylene iodonium, Trolox C (vitamin E analogue), and vitamin C inhibit VSMC growth, which is associated with pathogenesis of cardiovascular diseases. Therefore, inhibition of MAP kinases by antioxidant treatment may prove to be a therapeutic strategy for cardiovascular diseases. In contrast, some clinical studies have reported that antioxidant vitamins did not show beneficial effects in coronary artery disease or in a number of high-risk people. Thus, further studies are needed to clarify why antioxidants showed beneficial effects *in vitro*, whereas less satisfactory results were obtained in some clinical conditions.

Abbreviations: Ang II, angiotensin II; AP-1, activator protein-1; BMK1, big MAP kinase 1; DPI, diphenylene iodonium; EPR, electron paramagnetic resonance; ERK, extracellular signal-regulated kinase; H_2O_2, hydrogen peroxide; JNK, c-Jun N-terminal kinase; LOX-1, lectin-like oxidized LDL receptor-1; MAP, mitogen-activated protein; NAC, *N*-acetyl-*L*-cysteine; NADPH, nicotinamide adenine dinucleotide phosphate; $NO^{\cdot}$, nitric oxide; NOS, NO synthase; $O_2^{\cdot-}$, superoxide anion; ·OH, hydroxyl radical; $ONOO^-$, peroxynitrite; PDGF, platelet-derived growth factor; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; quercetin, 3,3´,4´,5,7-pentahydroxyflavone; Q3GA, quercetin 3-*O*-β-*D*-glucuronide; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; VSMC, vascular smooth muscle cell.

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OXIDATIVE STRESS AND CARDIOVASCULAR DISEASES

Because the phrase "oxidative stress" is currently very popular in many research fields, understanding its pathophysiological roles requires a clear definition of its meaning at the molecular level. While oxygen is an essential molecule for aerobic metabolism, it also has adverse properties that induce cell damage and cell toxicity. Through redox reactions, oxygen and nitrogen atoms are candidates for the production of free radical species during electron transfer in living systems. In general, oxidation and reduction reactions are linked. Thus, the loss of hydrogen atoms or electrons from one molecule (oxidation) and the acquisition of hydrogen atoms or electrons by another molecule (reduction) occur simultaneously. However, one electron reduction of oxygen produces the highly reactive free radical superoxide anion (Fig 1). Among the several products of oxidation-reduction reactions, oxygen-derived free radicals are called reactive oxygen species (ROS) and nitrogen-derived free radicals are called reactive nitrogen species (RNS). ROS include the superoxide anion ($O_2^{\cdot-}$), hydroxyl radical (·OH), hydrogen peroxide (H_2O_2), and lipid peroxides, while RNS include nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^-$).

Although the mitochondrial oxidation-reduction system and cytochrome P450 monooxygenase are well known generators of superoxide anion radicals, there are several other potential candidates for ROS generation, including xanthine oxidase, lipoxygenase, mitochondrial oxidase, NO synthase (NOS), and NADH/NADPH oxidase. Superoxide anion radicals are converted to H_2O_2 spontaneously or by superoxide dismutase (SOD). H_2O_2 is relatively more stable than other radicals and is one of the major intracellular ROS. While H_2O_2 is decomposed by catalase or peroxidase to water and oxygen, ·OH is generated via the Fenton or Haber-Weiss reactions.

Both ROS and RNS cause a variety of significant clinical and pathological effects. ROS are generated by neutrophils and macrophages during bacterial phagocytosis and by reperfusion following tissue ischemia. ROS are also generated by variety of other cell types, including vascular smooth muscle cells (VSMC), endothelial cells, and cardiomyocytes in the cardiovascular system[1-3]. Although nitric oxide is released from endothelial cells and has many beneficial effects against atherosclerosis through the dilation of arterial walls[4], NO^- react rapidly with superoxide anion radicals to form $ONOO^-$ within the vessels (Fig 1). $ONOO^-$ is involved in the development of atherosclerosis through lipid peroxidation and protein nitrosylation[5]. The apoptotic death of endothelial cells in the fibrous cap of the atherosclerotic legion appears to be involved in atherosclerotic plaque rupture through the activation of oxidized low-density lipoprotein receptor, LOX-1[6]. On the other hand, it is considered that endothelial cell proliferation benefits the repair process after arterial wall damage, while VSMC proliferation results in restenosis. It is

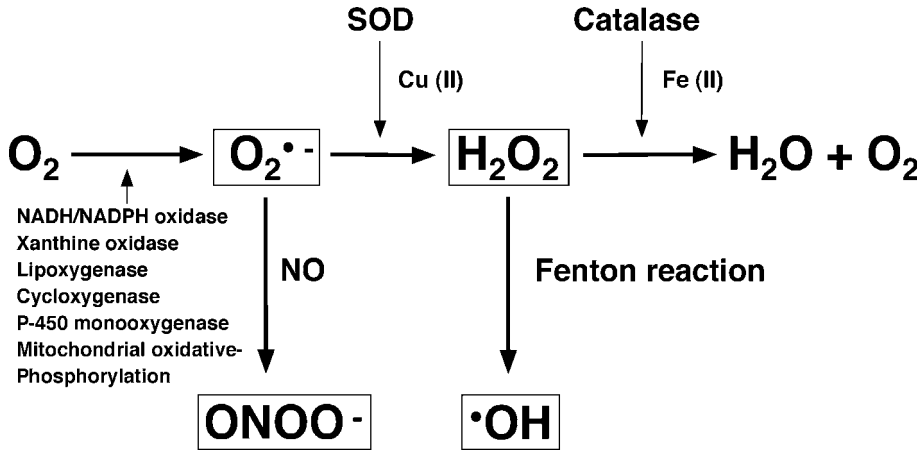

Fig 1. Sources of reactive oxygen species (ROS) generated endogenously by cardiovascular cells and key metabolic pathways for these species. Multiple enzymes may induce ROS generation in cardiovascular cells which these include NADH/NADPH oxidase, xanthine oxidase, lipoxygenase, cyclooxygenase, P-450 monooxygenase, and the enzymes of mitochondrial oxidative phosphorylation ($O_2^{\cdot-}$, superoxide anion radical; H_2O_2, hydrogen peroxide; ·OH, hydroxyl radical; $ONOO^-$, peroxynitrite; SOD, superoxide dismutase) (Cited from Ref 3 with permission).

now apparent that ROS induce endothelial cell damage and VSMC growth[1-3], which are responsible for cardiovascular remodeling, hypertension, atherosclerosis, heart failure, and restenosis.

BLOOD VESSEL WALL AND ROS

Recently, ROS including $O_2^{\cdot-}$ and H_2O_2 have been shown to be produced in the VSMC, endothelial cells and cardiomyocytes[1-3]. Many researchers have suggested that NADH/NADPH oxidase is a major source of ROS generation in the VSMC (Fig 2)[2,7]. The vascular NADH/NADPH oxidases share some similarities with the multisubunit enzyme complexes that comprise the neutrophil respiratory burst oxidases[8]. It has been shown that endothelial cells express the flavocytochrome b_{558} subunits gp91phox and p22phox, as well as the cytosolic factors p47phox and p67phox and the small G protein Rac-1[9-12]. All components of the neutrophil oxidase have also been found in adventitial cells[13], but so far only p67phox and Rac-1 have been shown to be functionally important[14,15]. In contrast, whereas VSMC contain p22phox[16] and p47phox[17], expression of p67phox and the catalytic moiety gp91phox have been difficult to be demonstrated[18]. It has been reported that multi-subunit containing NADH/NADPH oxidase are activated by growth factors such as PDGF and vasoactive peptides such as angiotensin II (Ang II) and endothelin[17-19]. It was also suggested that the role of p22phox, one of the major subunits of NADH/NADPH oxidase is important, by showing that p22phox antisense significantly inhibited superoxide generation induced by Ang II in VSMC[18].

Recently, nox-1 was cloned as a homolog of gp91phox, a subunit of NADH/NADPH oxidase in phagocytes[20]. It has been confirmed that nox-1 was also expressed in VSMC and preserves the main functional regions of gp91phox, the flavoprotein domain and the two heme binding domains[21]. Since anti-sense of nox-1 transfection inhibited the effect of Ang II-induced superoxide generation and serum-induced VSMC proliferation[20,22], it was suggested that nox-1 was an important subunit of NADH/NADPH oxidase in VSMC (Fig 2). In an electron paramagnetic resonance (EPR) study, we also demonstrated that Ang II and endothelin-1 stimulated ·OH radical generation in VSMC (Fig 3) and antioxidants inhibited these effects[23].

OXIDATIVE STRESS AND MAP KINASES

Mitogen-activated protein (MAP) kinases are protein serine/threonine kinases and play an important role in cell differentiation, growth, apoptosis, and the regulation of a variety of transcription factors and gene expressions. MAP kinases are activated by the phosphorylation of Threonine (T) and Tyrosine (Y) residues

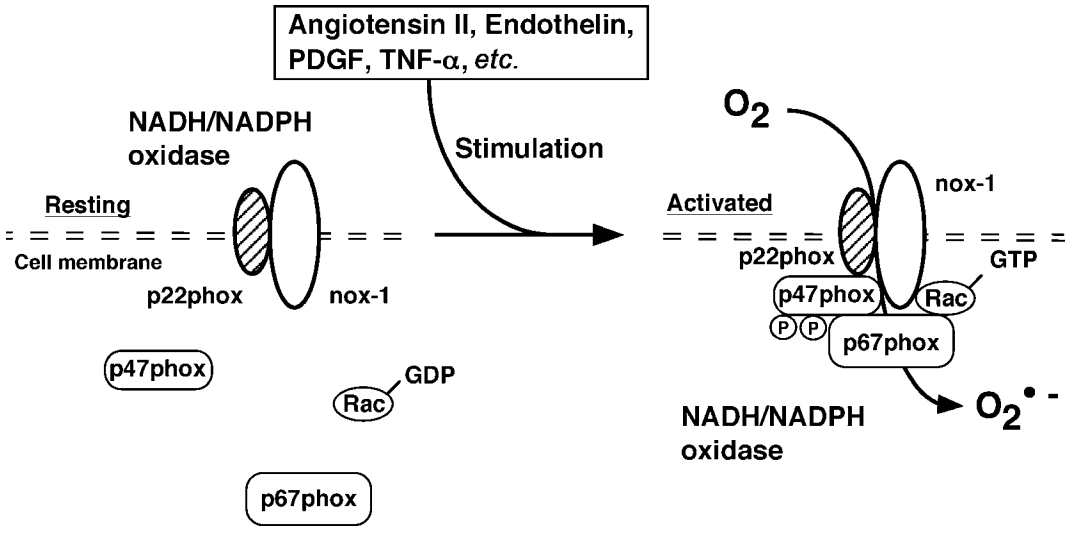

Fig 2. Structure and components of NADH/NADPH oxidase. Left panel shows the scheme of the resting state of the components. NADPH oxidase-1 (Nox-1) and p22phox form the electron transfer components of the oxidase, and p47phox and p67phox are cytosolic components that interact with these proteins to modulate its activity. The small G protein Rac also serves a regulatory function. The right panel shows the activated state of NADH/NADPH oxidase induced by agonists such as angiotensin II, endothelin, and PDGF (Cited from Ref 3 with permission).

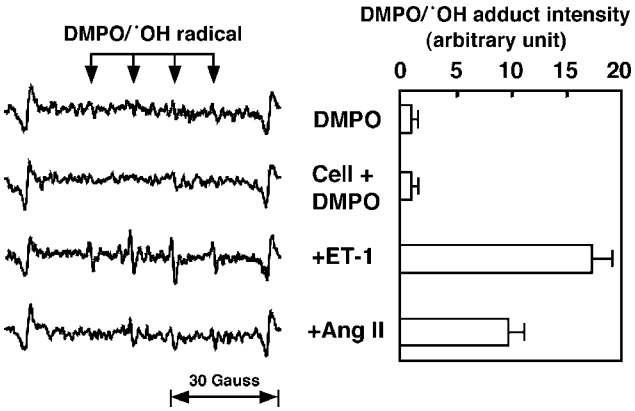

Fig 3. Effects of the ET-1- and Ang II-induced EPR signal of the DMPO/·OH spin adduct in rat aortic smooth muscle cells (RASMC). Representative EPR signals of the DMPO/·OH spin adduct are shown (left). Values are expressed as arbitrary units of the DMPO/·OH spin adduct signal intensity (right).

within a T-X-Y phosphorylation motif, where "X" can be Glu (E), Pro (P), or Gly (G). Three major classes of dual-specificity MAP kinases can be defined, based on their activation motifs (TEY, TPY, and TGY), which are termed extracellular signal-regulated kinase 1/2 (ERK1/2) and big MAP kinase 1 (BMK1) (also called ERK5), c-Jun N-terminal kinase (also called stress-activated protein kinase), and p38, respectively (Fig 4). Previously, it was reported that the ERK family mediates growth factor-stimulated cell differentiation and growth, and JNK and p38 mediate inflammatory cytokine- and stress-induced apoptosis and stress-responsive gene expression.

Several lines of evidence have indicated that ROS and MAP kinases are involved in vascular remodeling under various pathological conditions. Recently, these MAP kinases have been shown to have sensitivity to

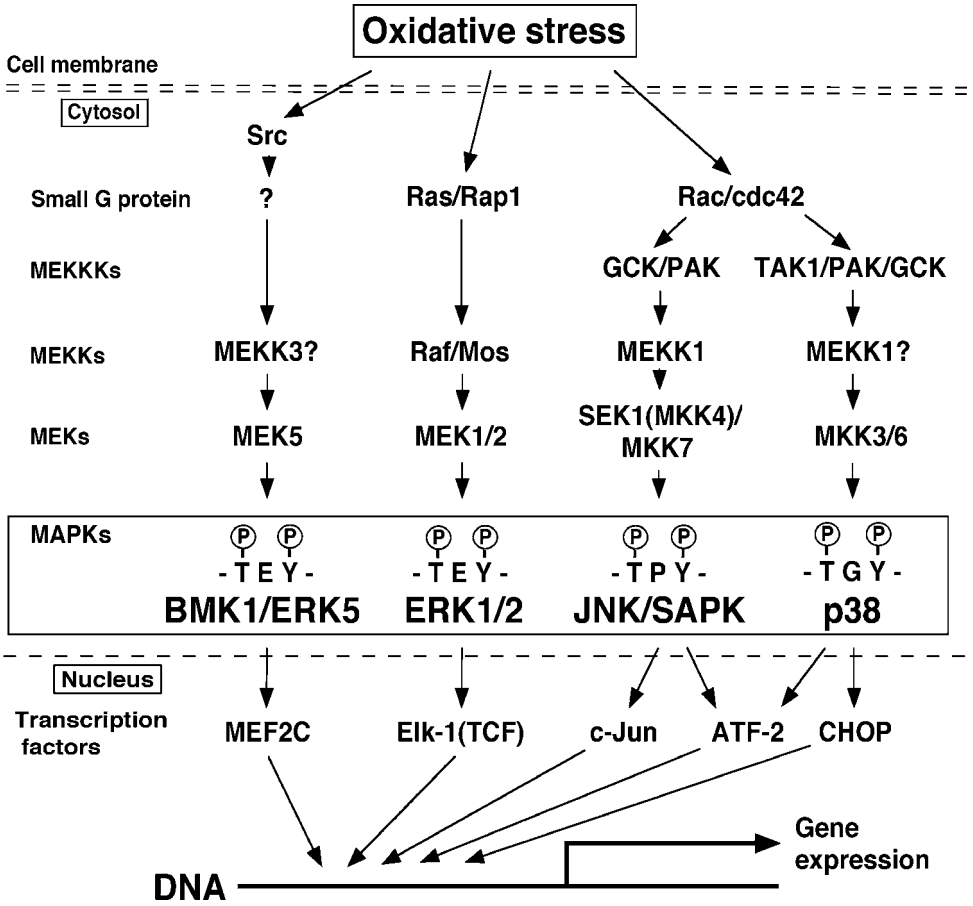

Fig 4. Signal transduction pathways for mitogen-activated protein kinases (MAPKs) by oxidative stress in cardiovascular cells. Shown in a highly schematic linear pattern, they are modules of kinases that regulate each other in a kinase cascade. For example, oxidative stress is proposed to activate the small G protein Ras and thereby stimulate MAPK and ERK kinase (MEK) kinase (MEKK) and subsequent activation of MEK1, a regulator of the MAP kinase ERK1/2. Additionally, it has been shown that other MAPKs including BMK1, JNK, and p38 may be regulated by oxidative stress. Among the targets for these MAP kinases are various transcription factors including Elk-1 (ternary complex factor, TCF), c-Jun, ATF-2, and CHOP (Cited from Ref 3 with permission).

oxidative stress. We also demonstrated that H_2O_2 rapidly and significantly stimulated MAP kinase family members ERK1/2, JNK, and p38 in rat aortic smooth muscle cells[24]. In this study, we found that JNK activation by H_2O_2 was dependent on the phosphorylation of Src tyrosine kinase and the Cas adaptor protein[24]. MAP kinase activation regulates cell differentiation, growth and apoptosis through phosphorylation of downstream target proteins and activation of transcription factors. Ang II and endothelin-1 are other activators of MAP kinases in VSMC and are believed to cause vascular remodeling[25,26]. Interestingly, Ang II potently activates ERK1/2 and p38; however, only p38 is sensitive to both inhibition of NADH/NADPH oxidase activity and catalase overexpression in VSMC[27,28]. We obtained similar findings that antioxidants preferentially inhibited JNK and p38 activation by Ang II but not ERK1/2 activation[29]. In our previous study, we found that vasoactive peptide endothelin-1 stimulated proliferation of human coronary artery smooth muscle cells through activation of ERK1/2 and transcription factor activator protein-1 (AP-1)[26]. Furthermore, we demonstrated that antioxidants inhibited endothelin-1-induced proliferation of VSMC via the inhibition of JNK and p38 which suggested the important role of ROS in MAP kinase activation and MAP kinase-mediated VSMC proliferation by endothelin[23]. It was also reported that MAP kinase mediated the increase in LOX-1 expression in endothelial cells[6].

EFFECTS OF FLAVONOIDS IN PREVENTATION AND TREATMENT OF ATHEROSCLEROSIS

As mentioned above, ROS-dependent oxidative stress is significantly involved in vascular remodeling associated with atherosclerosis through the induction of VSMC and fibroblast cell growth and endothelial cell apoptosis. Furthermore, we suggested that vasoactive peptides, Ang II and endothelin-1 acted as agonists to induce generation of ROS, and MAP kinases played an important role in the intracellular signaling mechanisms associated with vascular remodeling. Bioflavonoids, many of which are polyphenolic compounds, are believed to be beneficial for the prevention and treatment of atherosclerosis and cardiovascular diseases. Recently, much interest in flavonoids has been generated from the findings of the "French paradox"[30]. This paradox refers to the correlation of a high-fat and high-cholesterol diet with a lower incidence of coronary heart disease found in Mediterranean cultures contrasted with a higher incidence of coronary heart disease among most Western cultures. It has been shown that the French paradox may be attributable to regular consumption of red wine and that the unique antiatherogenic effects of red wine reside in the action of polyphenols.

Flavonoids are widely distributed in the plant kingdom and are categorized as flavonol, flavanol, flavanone, flavone, anthocyanidin, and isoflavone. 3,3´,4´,5,7-Penta-hydroxyflavone (quercetin) is one of the most widely distributed bioflavonoids, which are abundant in red wine, tea, and onions. Numerous *in vitro* studies have revealed diverse biological effects of quercetin, including apoptosis induction, antimutagenesis, protein kinase C (PKC) inhibition, lipoxygenase inhibition, histamine-release inhibition, superoxide dismutase (SOD)-like activity, modulation of cell cycles, angiogenesis inhibition, and inhibition of angiotensin converting enzyme[31]. Quercetin intake is therefore suggested to be beneficial for human health and its antioxidant activity should, at least in part, yield a variety of biological effects. The phenolic hydroxyl groups of flavonoids, which act as electron donors, are responsible for the compounds' free radical scavenging activity[32]. In particular, the catechol structure (*o*-dihydroxyl structure), which possesses two hydroxyl groups at neighboring positions, is remarkably superior to other conformations in electron donating ability and therefore quercetin and other flavonoids containing a catechol structure can exert powerful radical scavenging effects[32,33]. The antioxidant activity of flavonoids can also be explained by their chelating action, because transition metal ions such as the iron ion play a crucial role in the generation of ROS by Fenton-type reactions. In addition, the catechol group is recognized to contribute directly to the chelating action of flavonoids[33]. In fact, a number of studies have demonstrated that quercetin inhibits lipid peroxidation effectively by scavenging free radicals and/or chelating transition metal ions[34]. Recently, we demonstrated that quercetin inhibited Ang II-stimulated JNK activation through tyrosine phosphorylation of the adaptor protein Shc and activation of phosphatidylinositol 3-kinase (PI3-K) in cultured rat aortic smooth muscle cells (Fig 5)[35].

Flavonoids are mostly present as their glycosides in which one or more sugar groups are bound to phenolic groups by glycosidic linkage. Because of the difficulties in methodology, contradictory results have been reported as to the form of dietary quercetin present in

the circulation. Paganga *et al*[36] and Aziz *et al*[37] claimed that intact quercetin glycosides are present in human blood plasma. However, Manach *et al*[38] demonstrated that not quercetin glycosides but rather quercetin metabolites accumulated in the circulation at a concentration of 10^{-7}-10^{-5} mol/L after intake of a diet rich in quercetin glycosides. Moon *et al*[39] first succeeded in the isolation and identification of a quercetin metabolite, quercetin 3-*O*-β-*D*-glucuronide (Q3GA), in rat plasma after oral administration of quercetin aglycone. They also found that this metabolite possessed a considerable free radical scavenging activity because it contained a radical scavenging-catechol group. On the other hand, Wittig *et al*[40] reported the presence of five quercetin glucuronides in human plasma. These findings lead to two questions requiring further investigation in order to evaluate the biological action of dietary flavonoids. One is the necessity of identifying and evaluating the antioxidant activity of quercetin metabolites and the other is for elucidating the site and pathway of its metabolic conversion. In our study, we clearly showed that quercetin glucuronide as well as quercetin aglycone prevented Ang II-induced VSMC hypertrophy via the inhibition of the JNK and AP-1 signaling pathways (Fig 5)[41]. Another study reported that quercetin suppressed the inflammatory cytokine TNF-α-stimulated VSMC proliferation through the inhibition of ERK1/2[42].

Accordingly, these findings suggest that bioflavonoids have an anti-atherosclerotic effect. It was also reported that polyphenols extracted from red wine inhibited proliferation of VSMC[43]. In addition, resveratrol, a polyphenolic substance found in grape skin suppressed Ang II-induced activation of PI3-K/Akt pathway and subsequent hypertrophy of VSMC[44].

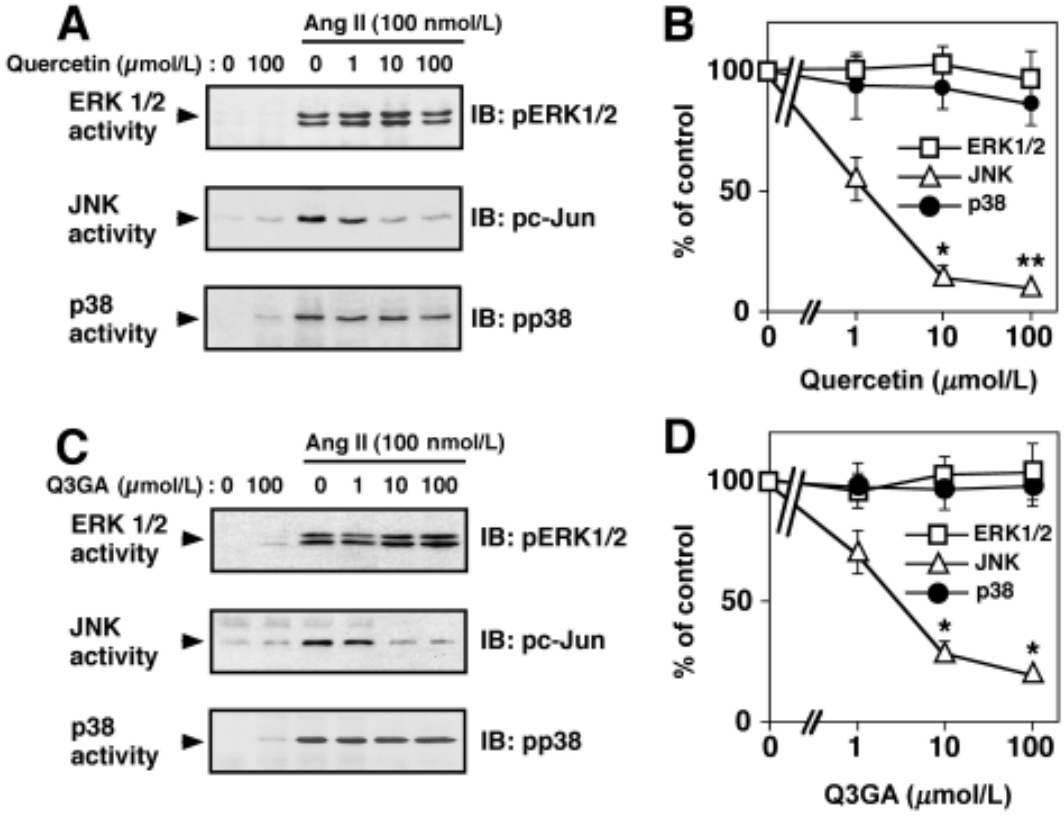

Fig 5. Inhibition by quercetin and Q3GA of Ang II-induced JNK activation, but not ERK1/2 and p38 activation, in a concentration-dependent manner, in rat aortic smooth muscle cells (RASMC). Cells were pretreated with quercetin and Q3GA at the indicated concentration for 30 min. Then, the cells were stimulated with 100 nmol/L Ang II for 5 min for ERK1/2 activity and 10 min for JNK and p38 activities. Cells were harvested and lysed. The activities of ERK1/2, JNK, and p38 were measured. A and C are representative blots. B and D shows the densitometric analysis of the effects of quercetin and Q3GA on ERK1/2, JNK and p38 activation. Values were expressed as percentages of control, which were defined from each MAP kinase activity stimulated by 100 nmol/L Ang II.

EFFECTS OF VARIOUS ANTIOXIDANTS ON ATHEROSCLEROSIS

A number of studies have been conducted to explore the roles of various antioxidants in cardiovascular diseases. Diphenylene iodonium (DPI), a potent inhibitor of a flavin-containing NADH/NADPH oxidase enzyme and overexpression of catalase enzyme, an H_2O_2 scavenger, has been shown to suppress p38 MAP kinase-mediated VSMC hypertrophy *in vitro*[28]. Furthermore, it was reported that *N*-acetyl-*L*-cysteine (NAC), a radical scavenger and intracellular glutathione precursor, and DPI inhibited endothelin-induced ROS generation, JNK activation and VSMC proliferation[19]. We also demonstrated that NAC and DPI as well as other antioxidants, Trolox C, a water-soluble vitamin E analogue, and vitamin C (ascorbic acid) abolished the stimulatory effect of Ang II on JNK and p38 activity in VSMC[29].

Clinically, antioxidants are believed to counteract ROS and reduce the incidence of coronary artery disease. Intake of vitamin E decreased the incidence of cardiovascular events in the population of ischemic heart disease patients in the Cambridge Heart Antioxidant Study (CHAOS)[45]. In addition, a multivitamins and probucol (MVP) trial revealed that probucol, an antioxidant, was effective in the prevention of restenosis after percutaneous transluminar coronary angioplasty (PTCA) procedures[46]. Recently, it has been reported that high dose vitamin C infusions improved endothelial dysfunction in patients with renovascular hypertension[47]. This study indicated that oxidative stress might partially be involved in hypertension. However, a GISSI-3 study[48] and HOPE study[49] could not show significant beneficial effects of vitamin E in the secondary prevention of coronary artery disease. A Heart Protection Study (HPS) in the UK also could not demonstrate any benefits of vitamin E, vitamin C, and β-carotene combined antioxidants therapy in a large number of high-risk people[50].

Accordingly, it was difficult to conclude that a clinical benefit of antioxidants in cardiovascular disease had been established. Thus, it is necessary to clarify why antioxidants showed their effects *in vitro*, whereas less satisfactory results were observed in some clinical conditions. In the future, it will be important to consider race differences and dose differences as well as the absorption and metabolism of antioxidants in performing *in vivo* experiments and clinical verifications.

FUTURE DIRECTIONS

As mentioned above, the implications of ROS and the role of MAP kinases in cardiovascular diseases have been well identified and characterized. In future studies, it will be necessary to prove the evidence clinically by confirming the effects of bioflavonoids *in vivo*. Also, further investigation of the pharmacology regarding absorption, distribution, metabolism, and excretion of antioxidants *in vivo* will be required. For a better understanding of the bench data to clinical applications, it is necessary to clarify the different mechanisms of antioxidants with *in vitro* and *in vivo* studies. The contradiction between *in vitro* and *in vivo* studies might be due to the much more complex situations *in vivo*. The selective antioxidants are thus putatively required for anti-atherosclerotic clinical trials.

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