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Resveratrol : natural properties against atherosclerosis, associated pro-inflammatory effects and aging

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Running title: Vascular protective effects of resveratrol

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1. Introduction

Vascular diseases including coronary heart disease (CHD), cerebrovascular and peripheral vascular diseases are the largest cause of mortality and morbidity in industrialized countries. Since many decades, various investigations have searched to identify the risk factors in cardiovascular diseases such as genetic factors, hypertension, and age. Some factors depend on our lifestyle such as smoking and diet. Indeed, diet high in fat and / or calories can lead to hypertriglyceridemia, a potent atherogenic risk factor. Besides a high-energy diet, certain unsaturated fatty acids may be pro-atherogenic and pro-inflammatory, some nutrients may protect against vascular diseases and associated inflammatory effects. A protective effect may be obtained with a diet rich in vitamin E [1], β -carotene [2], and in polyphenolic compounds found in fruits, vegetables and beverages. For example, in France, as compared with other western countries with a high-fat diet, the strikingly low incidences of CHD have been attributed partly to the consumption of red wine, which contains high levels of polyphenols [3]. Similarly, benefic effects may be attributed to the flavonoids of green tea. Indeed, several cohort studies demonstrate a significant inverse association between flavonoid consumption and cardiovascular risk [4]. The benefic effects of these compounds seem to be due to their antioxidant/antiradical activities protecting the vascular walls from oxidation, from inflammation, from platelet aggregation and thrombus formation. Vascular wall stiffening is also age dependent, due to in part to an enhancement of oxidative stress. Among the polyphenols with benefic properties, resveratrol, a phytoalexin of grape, reproduces the effect of a caloric restriction on the aging phenomena [5, 6]. Many studies evaluate resveratrol as a protective factor of degenerative diseases. Resveratrol possess a myriad of cardiovascular benefic effects and can act at multiple levels such as cellular signaling, enzymatic pathways, apoptosis and gene expression.

2. Resveratrol and atherosclerosis

The main cause of the coronary damages and particularly ischemic vascular diseases is the atherosclerosis. Briefly, the atherosclerotic process is the result of disruptions of normal reactions between the blood (plasmatic proteins, lipoproteins, growth factors, lymphocytes, platelets) and the normal cellular elements of the arterial wall. So, various compounds can be act at different cellular levels to brake the atherosclerotic lesion formation and these new anti-atherogenic drugs should be found in the diet. Indeed, various antioxidant compounds presents in food such as vitamin E, flavonoids and polyphenols, could be good candidates against atherosclerosis. Among this polyphenols, resveratrol could be a good agent acting at

different stages of physiopathologic atherogenesis (lipid accumulation and low-density lipoproteins (LDLs) oxidation; monocyte and lymphocyte infiltration; cellular smooth muscle proliferation and migration, platelets aggregation).

a) Resveratrol and lipoproteins

Target disruption of the apolipoprotein E (apoE) or low-density lipoprotein receptor (LDLR) genes, as well as overexpression of the human apolipoprotein B (apoB) gene in mice, result in marked increases in VLDL (very low-density lipoprotein) and /or LDL levels and subsequently contribute to atherosclerosis promotion [7]. In hypercholesterolemic mice (apoE^{-/-}/LDLR^{-/-}), resveratrol decreases the plasma lipid concentrations (total cholesterol and triacylglycerols) and reduces platelet aggregates [8]. The plasmatic concentration of lipids can also be reduced by the action of other apolipoproteins such as apoB or apolipoprotein I/II (apo I/II). So, resveratrol is able to reduce apoB content and secretion (which may be responsible for impaired LDL and VLDL synthesis) as well as the intracellular content and the rate of secretion of cholesteryl esters from hepatoblastoma cells [9, 10]. The rate of secretion of triglycerides (TGs) is also reduced by resveratrol, but the intracellular TGs content is unaffected. Taken together, these changes would tend to decrease the level of VLDLs which are rich in TGs and possess potential atherogenic properties (direct supply of cholesterol to fibroblasts; alterations of endothelial functions; transformation of monocytes-macrophages in foam cells). These events are found also *in vivo* in rats where resveratrol treatment decreases serum TGs, VLDL+LDL-cholesterol levels [11]. By its estrogenic similar structure, resveratrol could act on apoII. Indeed, hepatic expression of apoII is in part modulated by estrogen-mediated stabilization of its mRNA which is due to the estrogen-regulated mRNA stabilizing factor (E-RmRNASF). E-RmRNASF protect the RNA from target endonucleolytic degradation and its hepatic expression is modulated by estrogenic xenobiotics. Resveratrol seems to act as phyto-estrogens and it appears that resveratrol acts as an agonistic compound stimulating the E-RmRNASF expression [12]. These results suggest that resveratrol would have the capacity to modulate and block certain aspects of hepatic lipoprotein metabolism which predispose to atherosclerosis and the hypocholesterolemic action of resveratrol could be attributed to an increased excretion of neutral sterols and bile acids into feces.

b) Resveratrol and oxidative stress

The second important event in the lesion formation is LDLs oxidation in the intima [13, 14]. Lipid peroxidation is a chain reaction process which can be induced by different free-radical sources (ionizing irradiation, UV light). Several groups have reported that oxidized-LDL (ox-LDL) can stimulate platelet aggregation [15] or promote procoagulant activity in the

surface of human monocytes / macrophages by an increase in tissue thromboplastin activity [16] or by stimulating the expression and secretion of the tissue factor (TF) by monocytes or aortic endothelial cells [17].

Frankel et al, were the first to demonstrate that resveratrol added to human LDL, reduced the oxidation of human LDL induced by incubation with a heavy metal ion such as copper [18]. This effect should be assigned to the chelation of copper because metals act as pro-oxidants by electron transfer, releasing free radicals from polyunsaturated fatty acids and hydroperoxides. It has been demonstrated that resveratrol suppresses lipid peroxidation both by chelation of copper [19-21] and by scavenging of the free radicals [19, 20, 22]. The efficiency and action mechanism of *trans*-resveratrol have been demonstrated in the radical liposome oxidation where it appeared that *para*-hydroxyl group shows a greater radical-scavenging activity than *meta*-hydroxyl groups of *trans*-resveratrol [23]. Moreover, the spatial position of hydroxyl groups is likely more propitious to the chelation of copper in the *trans* isomer than in *cis* isomer [20]. Due to its hydroxylated structure, resveratrol can form a radical derivative stabilized by the delocalisation of two electrons between the two aromatic cycles and the methylene bridge joining these two cycles. In addition to metal ion induced oxidation of LDLs, various enzymatic systems presents in endothelial cells (ECs) or macrophages are implicated in the oxidation of LDL (figure 2). These systems include nicotinamide adenine dinucleotide (NADPH) oxidases, hypoxanthine / xanthine oxidase (HX/XO), 15-lipoxygenase (15-LO), myeloperoxidase (MPO) and nitric oxide synthases (NOS) [24, 25]. The products of these enzymes oxidize LDL which alter ECs, stimulate NADPH oxidase, the pro-inflammatory cytokines release, and inhibit endothelial nitric oxide synthase (eNOS) implicated in the vasorelaxation. So, resveratrol can act on these enzymes (figure 2).

NAD(P)H oxidases play an important role in superoxide production, O_2^- , in human vessels. Many cytosolic regulatory proteins (e.g. Rac) play an important part in regulating NAD(P)H oxidase activity in cardiovascular disease states by acute activation of the enzyme complex [26]. Resveratrol reduces the strain-increased NAD(P)H oxidase activity and NAD(P)H oxidase activity in rat aortic homogenates [27]. The isomer *cis*-resveratrol inhibits also NAD(P)H oxidase activity in macrophage homogenate [28]. These effects contribute to reduce intracellular reactive oxygen species (ROS) formation in EC caused by strain treatment.

Resveratrol inhibits leukocyte adhesion induced by other superoxide-dependent stimuli such as HX/XO which metabolize hypoxanthine, xanthine, and NADH to form uric acid, $O_2^{\cdot-}$ and H_2O_2 and platelet-activating factor [29].

Resveratrol is able to induce cellular antioxidants and phase 2 enzymes, including superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione-S-transferase, glutathione reductase, NADPH:quinone oxidoreductase [27, 29, 30]. These results are also found *in vivo*. These modifications contribute to increase the resistance to cardiac cell injury elicited by ROS.

Resveratrol reduced the generation of H_2O_2 , and normalized the levels of oxidized-glutathione reductase and MPO activities [31, 32]. MPO seemed to be important in vascular pathology because it change H_2O_2 to hypochlorous acid (HOCl) and other oxidizing species (figure 3). It also utilizes NO to generate ROS, thereby reducing NO bioactivity and increasing oxidative stress. By the normalization of the ROS levels, resveratrol limits the oxidative stress which inhibits NO synthesis by eNOS necessary for vasorelaxation (figure 3).

Oxidation induced by endothelial cells or by macrophages depends on lipoperoxides generated intracellularly and then transferred to the LDL. Cellular lipoxygenases, especially 15-lipoxygenase, appear to be involved [33, 34]. Various studies demonstrated that resveratrol inhibits lipoxygenases, in particular in human neutrophils where resveratrol strongly inhibits the 5- and 15-lipoxygenases producing in the arachidonate metabolism various proinflammatory products [35-38].

In addition to metal ions and ROS, ferrylmyoglobin and peroxynitrite are also potent oxidants implicated in oxidation of LDLs. Resveratrol was able to decrease the accumulation of hydroperoxides in LDL promoted by ferromyoglobin by reduction of the oxoferryl complex to metmyoglobin. Moreover the polyphenol inhibits LDL apoprotein modifications induced by peroxynitrite [39]. ROS production by polymorphonuclear leukocytes stimulated with formyl methionyl leucyl phenylalanine (fMLP) can be also strongly inhibited by resveratrol [40].

Moreover, resveratrol could act on targets in blood cells and in lipoproteins. Indeed, resveratrol was incorporated into blood cells and lipoproteins after *in vitro* incubations with plasma, lipoproteins and cells [41]. In fact, due to its lipophilic character, resveratrol is able to bind the lipoprotein particles suggesting that this event improved its anti-oxidant activity [42]. In lipoprotein particles, resveratrol is predominantly associated with their lipid moiety, but can be also associated with the protein moiety. Among plasma proteins, serum albumin could be involved [43]. This binding could explain that resveratrol reduce the oxidative alterations

of lipid and protein moieties of LDL [19]. By protecting apoB domains involved in the receptor activity of cells, resveratrol could reduce the non-specific uptake of oxLDL by macrophages.

c) Resveratrol and macrophages

In normal conditions, the monocytes enter, through diapedesis, the subendothelial space, where they differentiate into macrophages (figure 2). Under endothelial dysfunction, circulating monocytes adhere to the arterial endothelium, migrate to the subendothelial space, and differentiate into resident macrophages within the subendothelial matrix. OxLDL stimulate the expression of scavengers receptors CD36 and the class A scavenger receptor (SR-A) within monocytes, macrophages and smooth muscle cells (SMC) (which normally do not express this receptor). These receptors internalize the oxLDL in a specific manner, leading to a massive accumulation of cholesterol esters until foam cells are formed. These macrophage-derived foam cells make up the fatty streak that precedes more advanced sclerotic lesions (figure 2).

Oxidative stress caused by phorbol esters or reactive oxygen up-regulates the SR-A in human SMC, which normally do not express this receptor [44]. Resveratrol inhibits the activity and the expression of SMC cyclooxygenase-2 (COX-2) which normally produced prostaglandin E₂ (PGE₂) which up-regulate SR-A expression [44]. Various growth factors such as interleukin-1 (IL-1), tumor necrosis factor alpha (TNF α), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and transforming growth factor beta (TGF β) increase SMC SR-A activity [45]. Resveratrol could be able to decrease SMC SR-A activity through the action of these factors such as the decrease of EGF [46] (figure 2).

So, by the reduction of the interaction of oxLDL with macrophage scavenger receptors which play an atherogenic role, resveratrol contributes to prevent an early step in atherogenesis. At a molecular level, the acute formation of oxLDL-induced by ROS leads to the activation of mitogen-activated protein kinases (MAPK) pathways, which might be important for mitogenic signaling of oxLDL in VSMCs (see below figure 5). Resveratrol inhibits oxLDL-induced mitogenesis of VSMCs through the blocking of the ROS generation and the activation of the extracellular signal-regulated kinases (ERKs) pathway [47].

d) Resveratrol and foam cell formation

We have seen previously that oxLDLs favor the transformation of macrophages into foam cells [48]. The development of macrophage foam cells that contain massive amounts of cholesterol ester is a hallmark of both early and late atherosclerotic lesions. OxLDL derived cholesterol brought into the macrophage *via* scavenger receptors consists of free cholesterol

as well as cholesterol esters that are hydrolyzed in lysosomes. In addition, oxLDLs stimulate ECs to produce chemokines, granulocyte and macrophage colony-stimulating factors [49] and they have direct chemotactic activity for monocytes to endothelium [50]. Resveratrol contributes to reduce the production of chemokines which may be responsible for the chemotaxis and accumulation of macrophages in fatty streaks (figure 3). Resveratrol is able to inhibit interleukin-6 (IL-6) release by stimulated peritoneal macrophages in mice [51, 52], and in cortical mixed glial cells [53]. This action could result from a calcium blocking of calcium ion influx by resveratrol (see further “resveratrol and platelet aggregation”). Moreover, resveratrol contributes to reduce inflammatory response in atherosclerosis when macrophages (or SMC, EC) appear to be activated and produce numerous inflammatory products, such as TNF α , IL-6, monocyte chemoattractant protein-1 (MCP-1) (figure 3). Lesion progression is influenced by interactions between monocyte/macrophage and T cells. Lesional T cells appear to be activated, expressing both Th1 and Th2 cytokines [54] (figure 4). Resveratrol was able to inhibit the release of Th1-derived cytokines such as interferon γ (INF γ) which stimulates macrophage production of pro-inflammatory cytokines, IL-2 production by splenic lymphocytes and TNF- α and IL-12 production by peritoneal macrophage [55-57] (figure 4). The expression of mRNA encoding MCP-1 was also blocked by resveratrol [58]. Resveratrol was also able to inhibit Th2-derived cytokines such as IL-4 which exerts antagonistic effects on INF γ activity in macrophages and inhibition of Th1 cell function. Resveratrol inhibits the LPS-induced expression of IL-1mRNA in monocytes and ECs [59]. Concerning IL-8, the gene transcription as well as the protein production are inhibited by resveratrol [60].

This inhibition of cytokines production by the resveratrol is important for the regulation of adhesion molecule expression. Indeed, activated T lymphocytes and macrophages generate and release several cytokines with a number of biological effects on neighbouring cells [61]. So, various proinflammatory stimuli (e.g. interleukins, INF γ , TNF α , LPS) induce the expression of vascular adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1). These molecules mediate the firm adhesion of monocytes to the vascular endothelium in early atherosclerosis stages (figure 2 ou 3). Like others compounds of tyrophostine family which possess tyrosine kinase inhibitory activity [62, 63], resveratrol inhibits both the stimulated expression of VCAM-1 and monocyte adhesion to human vascular endothelial cells [64, 65]. These effects also affect E-selectin and ICAM-1. Indeed, resveratrol decreased significantly the expression of ICAM-1 and VCAM-1 induced on endothelial cells by TNF- α or lipopolysaccharide (LPS) [66], as well as neutrophile and

monocyte endothelial adhesion [67, 68]. This inhibition of adhesion molecule expression occurs at the same doses of resveratrol plasmatic concentrations ranging from 100 nmol/L to 1 μ mol/L in rat [64, 69]. It has been suggested that resveratrol may act as a rapid molecular signal interfering in the mechanism of VCAM-1 and ICAM-1 expression [70]. Vascular ECs can also be activated by proteolytic enzymes such as elastase which cause detachment or lysis of ECs and degradation of subendothelial matrices [71] and stimulate EC secretion of growth factors for SMC [72]. Resveratrol inhibits the release of both elastase and β -glucuronidase by polymorphonuclear leukocytes stimulated by fMLP and C5a and also inhibits their secretion [40]. So this modification of adhesion by resveratrol may support its use as an immunomodulating compound.

e) Resveratrol and vascular smooth muscle cells

Vascular smooth muscle cells (VSMCs) contribute to the pathogenesis of atherosclerotic lesions, since their proliferation and migration are critical events for progressive intima thickening and development of arterial wall sclerosis [73]. OxLDL can also promote the proliferation of the smooth muscle cells (SMC) which are in part resident intimal cells that preceded the lesions and in part their progeny that arose as a response to various stimuli (e.g. lipid accumulation, disruption of intimal structure). Intima SMC accumulate large amounts of cholesterol esters and become foam cells (figure 4). Inhibition of VSMC proliferation may have a beneficial effect in retarding development of atherosclerotic disease.

Resveratrol could delay atherogenesis by inhibition of VSMCs proliferation [74, 75]. Indeed, resveratrol is able to reduce SMCs proliferation induced by diverse mitogens such as serum, endothelin and PGDF. The antimitogenic effects of resveratrol are not mediated by the induction of apoptosis, but appear to be related to a G1 \rightarrow S block in cell cycle traverse [76, 77] and the DNA synthesis [75]. In fact, resveratrol leads to a reversible arrest in early S phase of the VSMC cycle. About the molecular mechanism, it exists a controversy: Haider et al have shown that the VSMC cycle arrest was accompanied by an accumulation of an hyperphosphorylated retinoblastoma protein, a decrease of cellular levels of the cyclin-dependent kinase inhibitors p21(Cip1), p27(Kip1), and an enhancement of phosphorylated of p53 protein [77]. On the contrary, Mnjayan and Fujise have shown that p21 and p53 are increased but this effect depends of resveratrol concentration [75]. Indeed, at lower concentration (6.25-12.5 μ M), resveratrol inhibits VSMCs proliferation without apoptosis described by Haider et al, but at higher concentration (25 μ M), resveratrol induces apoptosis in serum-stimulated VSMCs but not in quiescent VSMCs. These results suggest that resveratrol may be able to selectively eliminate abnormally proliferating VSMCs of the arterial walls *in*

in vivo. Resveratrol can also inhibit VSMCs proliferation induced by AGEs (Advanced Glycation End-product) of plasma proteins and/or matrix proteins which are mediators implicated in various vascular complications [78]. AGEs increase coagulation through various mechanisms involving the vascular endothelium and platelet activation [79]. AGEs also increase DNA synthesis and propyl hydroxylase activity, a marker of collagen synthesis in stroke-prone spontaneously hypertensive rats (SHRSP) or Wistar-Kyoto rats (WKY) VSMCs. These phenomenon are inhibited by resveratrol in animal experimental model [80]. In this same perspective of fighting against atherosclerosis process, it has been shown that the inhibition of pulmonary artery endothelial cells proliferation by resveratrol is correlated with the suppression of cell progression through S and G2 phases of the cell cycle [81, 82].

f) Resveratrol and vasorelaxation

Resveratrol was able to inhibit the production of endogenous vasoconstrictors and thereby regulates vasomotion which is impaired in atherosclerosis. The key regulators of vasomotor function are the vasodilator NO and the vasoconstrictor endothelin-1 (ET-1). In VSMC, oxidative stress increases the ET-1, which is involved in endothelial dysfunction, generation and autocrine ET-1 activity. Resveratrol inhibits strain-induced ET-1 secretion [83, 84], ET-1 mRNA level, and ET-1 promoter activity [84]. This inhibition of strain-induced ET-1 gene expression was partially due to resveratrol attenuation of activator protein 1 (AP-1) binding activity and resveratrol interference in the ERK1/2 pathway through attenuation of ROS formation [84] (figure 5). Resveratrol inhibits ET-1 surproduction and cytosolic phospholipase A₂ (PLA₂) activity stimulated by oxidative stress [83]. ET-1 expression can be induced by several substances such as angiotensin II (Ang II), thrombin, PDGF-A, and TNF α [85]. So, resveratrol can reduce ET-1 expression by its action on the latter factors. Indeed, resveratrol can act on Ang II. Angiotensin II-induced hypertrophy of vascular VSMCs is a pivotal step in the development of CHDs. Resveratrol could fight angiotensin II (Ang II)-induced VSMC hypertrophy by interfering with the phosphatidylinositol 3-protein (PI3K)/Akt and p70 ribosomal protein S6 kinase (p70(S6K)) [77, 86]. Indeed, resveratrol is able to attenuate the phosphorylation of p70(S6K) as well as the phosphorylation of Akt/ protein kinase B (PKB) and ERK1/2, both essentially involved in Ang II-mediated hypertrophy (figure 5). This action on Ang II by resveratrol can protect from cardiac fibrosis. Indeed, cardiac fibrosis results of a prolonged activation of cardiac fibroblasts (CFs) leading to a reduction of myocardial contractile function. Resveratrol inhibits Ang II-induced ERK1/2 and ERK kinase activation in CFs [87]. Moreover, pretreatment of CFs with resveratrol reduced both Ang II- and TGF β -induced CF differentiation to the myofibroblast phenotype, indicated

by a reduction in alpha-smooth muscle actin expression and stress fiber organization in CFs. So, resveratrol appeared to act as an anti-fibrotic agent in the myocardium. Furthermore, the reduction of Ang II concentrations would reduce the increase of NADPH oxidase-derived ROS.

ET-1 activates specific receptors, designated as ET_A and ET_B [88]. So, resveratrol by its action on PLA₂ and other signalling pathways appears to protect against VSMC contraction mediated by the ET_A-receptor.

The inhibition of strain or the induction of vasorelaxation can also be dependent on NO production, Na⁺ concentrations or cGMP pathways. For the NO production, it has been clearly documented that resveratrol can modulate the level of NO by its action on both eNOS and iNOS. Under normal conditions, ECs produce NO at a low level to control vessel dilatation. However, in atherosclerosis, a high level of NO has been found within early lesions and advanced atheroma even though expression of eNOS is reduced. On the contrary, the inducible Ca²⁺-independent NOS, also known iNOS, was increased. It has been shown that resveratrol can cause NO-mediated relaxation of precontracted endothelium-intact rat aorta through an increase of NO *via* eNOS [89-93]. At molecular level, resveratrol enhanced eNOS expression and inhibited iNOS expression by an action on their promoter (see below resveratrol and nuclear targets). In fact, resveratrol increases the activity of the eNOS promoter and eNOS mRNA stabilisation [94]. The vasorelaxation mediated by the polyphenol was reversed by the constitutive Ca²⁺-dependent NOS (cNOS). The compound also induces a NO-independent vasodilatation on denuded aorta, and the vasorelaxative activity of resveratrol depends also on direct stimulation of K⁺/Ca²⁺ channels in ECs [95]. So, it seems that the ability of resveratrol to modulate calcium channels in ECs could contribute to control the vasorelaxation mediated by nitric oxide (NO) (see below resveratrol and platelet aggregation).

Concerning the cGMP pathway, resveratrol increases cGMP in coronary arteries, mostly by activation of pGC [96]. Resveratrol activates membrane-bound guanylate cyclase GC-A, the receptor for atrial natriuretic factor (ANF) [97]. At molecular level, the cGMP/kinase-G is an antiproliferative signaling in SMCs and it dilates blood vessels through the reduction of intracellular calcium. The cytotastic actions of cGMP in SMCs involved apoptosis, inhibition of PI3K and mitogen-activated protein kinases (MAPKs) interfering with the cell-cycle machinery [98]. So the activation of pGC by resveratrol triggers vasorelaxant responses that remain effective in endothelium-disrupted arteries.

Resveratrol could also influence the vasorelaxation through an action on the activity of BK(Ca) channels which are functionally expressed in vascular ECs; it controls K⁺ efflux and affects intracellular Ca²⁺ concentration. In fact, resveratrol opens large and small conductance Ca²⁺-activated K⁺ (BKCa) channels, but not ATP-sensitive K⁺ channels [99] and increases the activity of large conductance BKCa channel in ECs [95, 100]. The resveratrol-stimulated increase in the channel activity was independent of internal Ca²⁺. So, the increase in K⁺ efflux through resveratrol-induced stimulation of KCa channels in ECs may contribute to produce vasorelaxation.

g) Resveratrol and angiogenesis

Angiogenesis is important in atherosclerosis where EC migration, proliferation are essential events in this process. Vascular endothelial growth factor (VEGF) co-localizes with endothelial cells, macrophages and SMC in atherosclerotic plaques [101]. Resveratrol inhibits VEGF-induced angiogenesis by abrogating VEGF-mediated tyrosine phosphorylation of vascular-cadherin and its complex partner, β -catenin [102]. The inhibition of VEGF-induced angiogenesis is mediated by the disruption of ROS-dependent Src kinase activation and the subsequent VE-cadherin tyrosine phosphorylation. Resveratrol can also reduce VEGF by its action on NADPH oxidase [27, 28] which regulates the induction of VEGF expression [103] and the VEGF-induced angiogenesis [104]. VEGF expression can be also regulated by pro-apoptotic factors such as AngII, which may be accumulated in response to ECs damage. Consequently to its effect on Ang II, resveratrol can inhibit Ang II-mediated VEGF expression [86]. Furthermore, it inhibits both the FGF (fibroblast growth factor) receptor- and the VEGF receptor-mediated EC responses [105].

h) Resveratrol and platelet aggregation / thrombosis

Platelets contribute to the rate of development of atherosclerosis and CHD through several mechanisms. It has been shown that resveratrol reduces platelet aggregation in human platelet-rich plasma in particular after induction by thrombin and adenosine-5'-diphosphate (ADP) treatment [106-108]. These *in vitro* results can were found again *in vivo* [109]. In fact, thrombin downregulates endothelial ectonucleotidase activity resulting in high level of ADP and ATP which lead to platelet and endothelial activation. Resveratrol inhibits thrombin-induced ADP and ATP secretion from platelets, decreases neutrophil function and restores the CD39/ATPDase (ATP diphosphohydrolase) in response to thrombin [110]. Furthermore, when activated by thrombin, platelets produce ROS. This free radical generation can be reduced by resveratrol in blood platelets [111, 112]. In addition to thrombin and ADP another factor, platelet-activating factor (PAF), has been reported to be also involved in atheromatosis

generation. Resveratrol was able to inhibit PAF-induced platelet aggregation [113] and its pro-inflammatory effects [29]. The PAF-induced platelet aggregation is accompanied by the release of thromboxane A₂ (TxA₂), a pro-aggregant and vasoconstrictor agent. Moreover, PAF stimulates polymorphonuclear leukocytes to aggregate, to release leukotrienes and to generate superoxide. Similarly, PAF promotes aggregation of monocytes. So, resveratrol inhibiting PAF effects can reduce the effects of pro-aggregants / pro-inflammatory agents such as eicosanoids and leukotrienes.

The synthesis of eicosanoids and leukotrienes from arachidonic acid is also linked to the platelet aggregation. The synthesis of products from arachidonic acid in human platelets occurs according to several pathways. The lipoxygenase pathways lead to hydroperoxyeicosatetraenoic acids (HPETE) which are unstable and may be converted to their corresponding hydroxy fatty acids (HETE). The cyclooxygenase (COX) and the prostaglandin H synthase (PHS) pathways lead to the cyclic endoperoxides and the subsequent metabolic products such as TxA₂.

Resveratrol is able to act on lipoxygenase family. Resveratrol inhibits both 5-lipoxygenase and 15-lipoxygenase as a competitive inhibitor [38]. Resveratrol prolonged the lag phase of both enzymes, indicating a possible reduction of Fe(III) to Fe(II) at the catalytic site [114]. Pinto et al have shown that resveratrol inhibits the dioxygenase activity of lipoxygenase and is simultaneously oxidized by the peroxidase activity of lipoxygenase. The oxidized form of resveratrol is a lipoxygenase inhibitor as efficient as the reduced form [37, 115]. This lipoxygenase inhibition by resveratrol prevents the release of pro-inflammatory substances such as 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid (5,12-diHETE), 12-hydroxyeicosapentaenoic acid (12 HETE), leukotriene B₄ (LTB₄) and its isomers (6-*trans*-LTB₄, 12-*trans*-epi-LTB₄) and its glutathione-conjugated derivative (LTC₄) [35, 40, 116]. The 12-lipoxygenase pathway of arachidonate metabolism, which is present in leukocytes and platelets, can be reduced by resveratrol, which blocks the synthesis of heparin, mediators of calcium mobilization, vascular permeability and neutrophil activation [117, 118].

Resveratrol is also a competitive inhibitor of COX and peroxidase activity of PHS [38]. As far as PHS is concerned, both COX and peroxidase activities depend on ferriprotoporphyrin IX [119, 120]. Again, the prolonged lag phase of the COX reaction was indicative of a reduction of Fe(III) to Fe(II) [120, 121]. The COX inhibition by resveratrol prevents the release of COX products such as prostaglandins and thromboxanes. For example, the polyphenol reduces the prostaglandin synthesis, decrease HHT, TxA₂ as well as TxB₂

synthesis which are proaggregant and vasoconstrictor agents [35, 118, 122, 123]. By inhibition of PLA₂, resveratrol decreases the release of arachidonate from cell lipids and thus the synthesis of metabolites by COX and lipoxygenase pathways [123]. Moreover, the polyphenol could act on the Ca²⁺ influx, subsequently reducing the activation of PLA₂ and the aggregation process. Indeed, an increase in intracellular free Ca²⁺ is an essential component of the aggregation process in platelets. Ca²⁺ must enter the cell from the external media through specific and tightly regulated Ca²⁺ channels in the plasma membrane. It appears that resveratrol is an inhibitor of store-operated Ca²⁺ channels and calcium influx in thrombin-stimulated human platelets [124-126]. Moreover, the blocking of calcium ion influx into cultured murine macrophages by resveratrol is one of the possible mechanisms of the pro-inflammatory IL-6 biosynthesis inhibitory action of resveratrol [51]. Nevertheless, Slater et al. [127] found that the inhibition of PKC α activity is competitive with respect to phorbol ester concentration but non competitive with respect to Ca²⁺ and phosphatidylserine concentrations suggesting that resveratrol compete for phorbol ester binding site to the C1 domains.

Ca²⁺ regulates various pathways and is a major second messenger implicated in signal transduction pathways regulating cell cycle, proliferation and apoptosis. Several pro-atherogenic stimuli induce ECs apoptosis through Ca²⁺-dependent pathways and contribute to the development of vascular lesions. OxLDL-mediated endothelial cells apoptosis is dependent on an increase in intracellular Ca²⁺ [128]. Thus, alterations in intracellular Ca²⁺ in ECs may cause EC dysfunction in response to oxLDL and may influence EC response to oxLDL and inflammatory cytokines, particularly TNF- α . Resveratrol blocking the Ca²⁺ influx could prevent the EC apoptosis. Several matrix elements play also an important role in platelet aggregation such as collagen and fibrinogen. Resveratrol was shown to block the first step of platelet activation by inhibiting platelet adhesion to type I collagen and to decrease collagen-induced platelet aggregation [129, 130] (figure 4). Moreover, resveratrol inhibited the messenger RNA (mRNA) expression of type I collagen [131]. At cellular level, in the platelets, resveratrol can inhibit MAPK activation induced by collagen, thrombin and ADP [132] (figure 5). So, resveratrol could block receptor-mediated signaling events in platelets.

Concerning the blood platelet adhesion to fibrinogen, another initial step of platelet activation, resveratrol inhibits adhesion of both thrombin- and ADP-activated platelets to fibrinogen [133] or after activation by LPS or LPS with thrombin [130, 134]. Moreover, resveratrol could protect against atherosclerosis by promoting fibrinolysis. Indeed the polyphenol such as others compounds (catechin, epicatechin) is able to up-regulate both

tissue-type plasminogen activator (t-PA) and urokinase-type PA (u-PA) gene transcription which are fibrinolytic proteins [135].

Thrombosis plays a critical role in the development, progression and clinical after-effects of atherosclerosis. The primary initiator of thrombus that mostly consisted of platelet aggregates, is a cell surface receptor for factor VII (a), the tissue factor (TF) [136]. The reduction of TF expression in vascular cells, ECs and monocytes may also contribute to the anti-aggregatory effects of resveratrol [59, 137] (figure 4). In fact, resveratrol reduced TF activity, TFmRNA by inhibition of nuclear factor kappa B (NFκB) / Rel-dependent transcription by impairing the transactivation potential of p65 [137, 138]. The diminution of c-Rel/p65 activity was dependent upon inhibition of degradation of the c-Rel/p65 inhibitory IκBα (inhibitor of κB) [138] (figure 5). The anti-thrombosis properties of resveratrol have been also shown *in vivo*. Indeed, resveratrol orally administered with a high-fat diet in genetically hypercholesterolemic mice (apoE^{-/-}/LDLR^{-/-}) suppressed the formation of atheroma in the aortae and reduced the laser-induced thrombosis in their carotid arteries [8].

i) Resveratrol and cellular / nuclear targets

Specific non-antioxidant effects of resveratrol in cellular signaling and regulation of gene expression have been studied and have an important impact on atherosclerosis development.

Resveratrol was able to act on the MAPK cascade. Downstream targets for the action of MAPKs comprise mitogenic/pro-inflammatory enzymes and nuclear transcription factors (figure 5). Resveratrol is able to act at different levels. Indeed, resveratrol is able to act on upstream pathway by inhibiting the phosphorylation and the activity of PKC [127, 139, 140]. Resveratrol inhibits PKC-catalyzed phosphorylation of arginin-rich protein substrate in a non competitive manner [141]. The potency of resveratrol depends on the nature of the substrate and cofactors [141]. As diacylglycerol, resveratrol interacts with the C1 domains and induces the association of PKCα with membrane vesicles. Resveratrol can also inhibit other kinases such as Src which activates MAPK cascade [102]. Resveratrol inhibits also the PI3K phosphorylation and prevents the Akt / protein kinase B (PKB) phosphorylation. Consistent with this action, resveratrol attenuates the phosphorylation of p70^{S6K} which was shown in VSMC to require both the Akt / PKB and the ERK signaling cascades [86]. Consequently, resveratrol disturbs the protein synthesis because p70^{S6K} plays a critical role in regulating the translation of mRNAs. Resveratrol downregulates MAPK cascade by inhibiting the tyrosine phosphorylation of ERK1/2/JNK/p38 and the translocation into nucleus in the vascular cells

[47, 84, 142]. This inhibition of phosphorylation and of translocation into nucleus from cytoplasm, reduces the expression of various genes implicated in the vasoconstriction, angiogenesis, proliferation, differentiation. In addition to its action on MAPK cascade, the polyphenol affects nuclear factors and consequently the gene expression. It affects NF κ B which activates the transcription of several target genes implicated in initiation and progression of pathogenesis in atherosclerosis, in inflammation, as well as in cancer [143]. Many stimuli such as oxLDL, ROS, PKC, have the potency to activate the NF κ B pathway. NF κ B is located in the cytoplasm as an inactive complex when associated with the inhibitor of κ B (I κ B). In response to stimuli, the catalytic subunits of I κ B kinase (IKK) complex phosphorylate I κ B α at two conserved serines. This phosphorylation event triggers the ubiquitin-dependent degradation of I κ B by the 26S proteasome. Active p50/p65 complex is subsequently activated by phosphorylation of IKK α and PKC resulting in nuclear translocation of p50/p65 heterodimers (figure 5). The nuclear NF κ B then binds to specific κ B DNA motifs and modulates the transcription of target genes (e.g. COX, iNOS, cytokines, ...).

The first study on the effect of resveratrol on NF κ B showed that treatment with oxLDL and VLDL activate NF κ B binding activity and that resveratrol attenuates the activation of NF κ B in PC-12 cells [144]. Furthermore, thanks to its properties of ROS scavenger and PKC inhibitor, resveratrol blocks stimuli-mediated phosphorylation and degradation of I κ B α as well as the activation of IKK α (figure 5) [58, 145, 146]. Resveratrol inhibits the phosphorylation of p65 and its transactivation [137] by inhibiting kinases such as IKK α [145], PKC [127] and the intrinsic kinase of PKC δ [147]. A recent study shows that a long treatment with resveratrol in human umbilical vein endothelial cells increases tyrosine phosphorylation of I κ B α , p50-NF κ B and p65- NF κ B suggesting the involvement of such alterations in the modulation of NF κ B transcription activity [148]. It has been also reported that resveratrol is a potent inhibitor of NF κ B nuclear translocation and I κ B degradation [58]. Resveratrol blocks the translocation of the p65 subunit of NF κ B in inflammatory agents (TNF α , PMA, LPS, H₂O₂)-stimulated cells resulting in reduced transcriptional activity [146]. GATA and AP-1 are also affected by resveratrol. Indeed, the suppression of NF κ B by resveratrol coincides with the inhibition of activator protein-1 (AP-1) [149]. In fact, resveratrol inhibits stimuli-induced AP-1-mediated activity [146, 150, 151] through the inhibition of c-Src non-receptor tyrosine kinase [152] and MAPK pathways such as MEKK1 and JNK [146, 152], which can activate both AP-1 and NF κ B pathways [153, 154].

Moreover, resveratrol reduces the DNA binding activity and transcriptional activities of AP-1 and its composition [60, 84, 155]. The disturbing of the nuclear factors (e.g. NFκB, AP-1, GATA,...) by resveratrol affects the genes expression. In particular two genes, iNOS and COX-2, are involved in the CHD process. Concerning the iNOS gene, its expression is controlled, in part, by NFκB [156]. So, resveratrol is able to inhibit iNOS expression in various cell types [157-162], in particular in macrophages regulating blood pressure where resveratrol inhibits iNOS and down-regulates NFκB [161]. Concerning COX-2, various reports demonstrate the presence of COX-2 expression by SMCs in human atherosclerotic lesions [163, 164], and its expression is also regulated by various nuclear factors such as NFκB, AP-1, c-Jun [165]. Many studies demonstrated that resveratrol inhibits COX expression *via* an action on the nuclear factor such as AP-1, c-Jun [150, 151, 166, 167]. Moreover, docking studies on both COX-1 and COX-2 protein structures also revealed that hydroxylated but not methoxylated resveratrol analogues are able to bind to the previously identified binding sites of the enzymes [168]. This down-regulation of COX-1/2 genes expression by resveratrol is correlated with a decrease of inflammation [122]. Indeed, the inflammatory aspect of atherosclerosis include the COX-dependent prostaglandin cascade, and so resveratrol decreases the level of prostaglandin by a reduction of COX-2 activity. Resveratrol can also act on COX-1/2 *via* the peroxisome proliferator-activated receptor (PPAR). In human VSMC a PPARα agonist has been shown to decrease NFκB activity [169]. Decreased NFκB activity reduces COX-2, so PPARα can depress the COX-1/2 induction in human. Activation of this PPAR may contribute to the anti-inflammatory activity of the pharmacological ligands that influence the development of atherosclerosis [170]. Resveratrol is able to activate PPARα in vascular EC and its consumption (20 mg/kg, 3 days) reduces infarct volume by 36% at 24 hours after middle cerebral artery occlusion in mice [171]. In fact, resveratrol is a dual activator of PPARα and PPARγ [171]. So, by this activation of PPARs, resveratrol could contribute to the lipid metabolism modulation and prevent the inflammatory activation of SMCs. Moreover, PPARα shifts the human liver fatty-acid oxidation/glycerolipid esterification balance towards the catabolic route, thereby reducing TGs supply for VLDL synthesis and contributing to the antihypertriglyceridaemic action of resveratrol.

By attenuation of nuclear factors binding activity (e.g. NFκB, AP-1, GATA), resveratrol perturbs the control of the expression of various genes (e.g. ET-1, MCP-1, VCAM-1, ICAM-1, SR-A, IL-1, IL-6) involved in atherosclerosis and inflammatory response [58, 65, 84, 172].

Caloric

Among the polyphenol with benefics properties, resveratrol, a phytoalexin of grapewine, can acts on the aging phenomena as well as caloric restriction which reduces atherosclerosis [173], inflammation [174] and delecters effects of aging [175].

Conclusion

In VSMCs, Ang II and TNF α were shown to induce these genes through the activation of NF κ B in a ROS-dependent manner [176, 177].

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Legends of figures

Figure 1: Chemical structure of resveratrol (3,5,4'-trihydroxystilben in classical nomenclature).

Figure 2: Resveratrol effects on initial events of atherosclerosis. Resveratrol (R) prevents the initial events by scavenging the ROS (✱), by inhibition of the enzymatic systems producing ROS (NADPH oxidase, Hypoxanthine / xanthine oxidase (HX/XO), cyclooxygenase (COX)), by downregulation of scavenger receptor (SR-A) stimulated by several factors, by induction of e NOS involving a vasorelaxation.

Figure 3: Resveratrol effects on chemokine production. Resveratrol (R) decreases significantly the expression of ICAM-1 and VCAM-1 induced on endothelial cells by TNF- α or lipopolysaccharide LPS, as well as neutrophile and monocyte endothelial adhesion. Resveratrol can also inhibits iNOS as well as MPO secreted from macrophages and so reduces the endothelial activation- and inflammation-mediated by oxLDLs. The polyphenol can reduce the apoptosis of ECs induced by TNF α and AngII.

Figure 4: Resveratrol effects on advanced atherosclerotic lesion formation. Resveratrol (R) blocks the cytokine production and reduces the SMC proliferation and migration. Furthermore, resveratrol inhibits paletelet aggregation as well as pro-aggregants/ pro-inflammatory agents (eicosanoids, leukotrienes) and subsequently inhibits the formation of a thrombus.

Figure 5: Resveratrol effects on signaling pathways. Resveratrol inhibits VEGF-induced angiogenesis by disruption of ROS-dependent Src kinase activation. By this action on Src and MAPk cascade, resveratrol inhibits angiogenesis and the translocation of nuclear factors into the nucleus from the cytoplasm. Moreover, resveratrol exogenous oxidants (oxLDLs, ROS), calcium flux and synthesis of pro-inflammatory compounds. The polyphenol can also inhibit stimuli involved in the activation of NF κ B pathway such as PKC α signal transduction, I κ B phosphorylation, IKK activity, p50/p65 nuclear translocation. These effects on the signaling pathways lead to a down-regulation of many gene transcription (COX-2, iNOS, VACM, SR-A,...) involved in atherosclerotic process and inflammation.

Figures

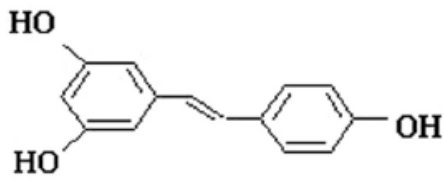


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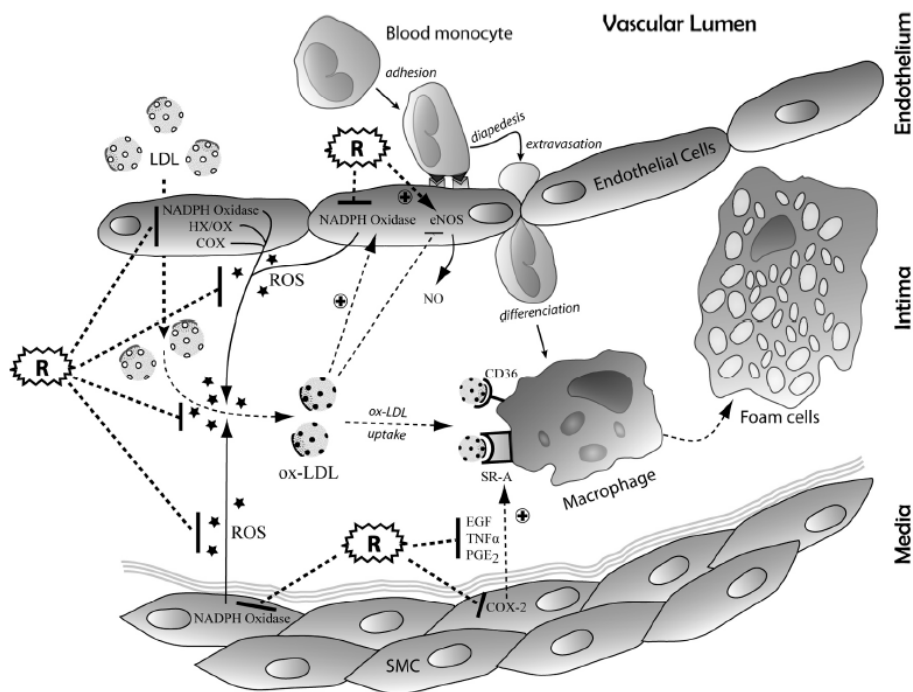


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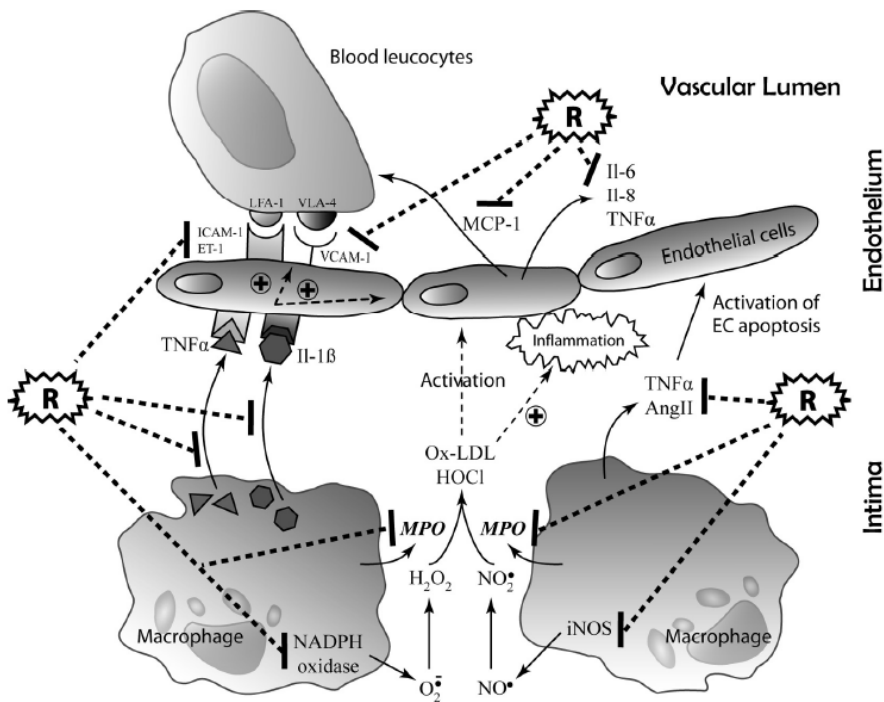


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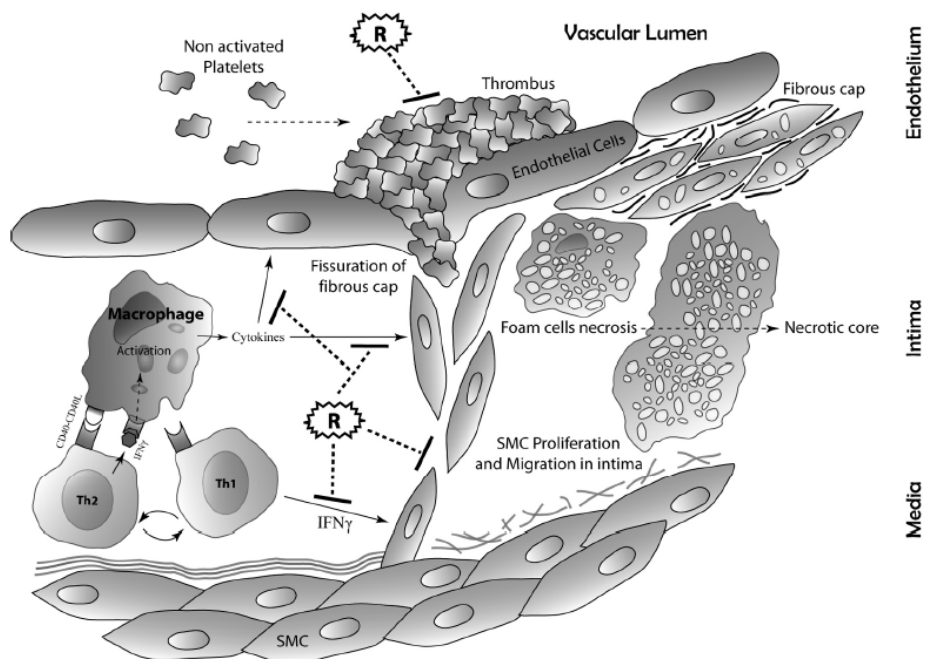


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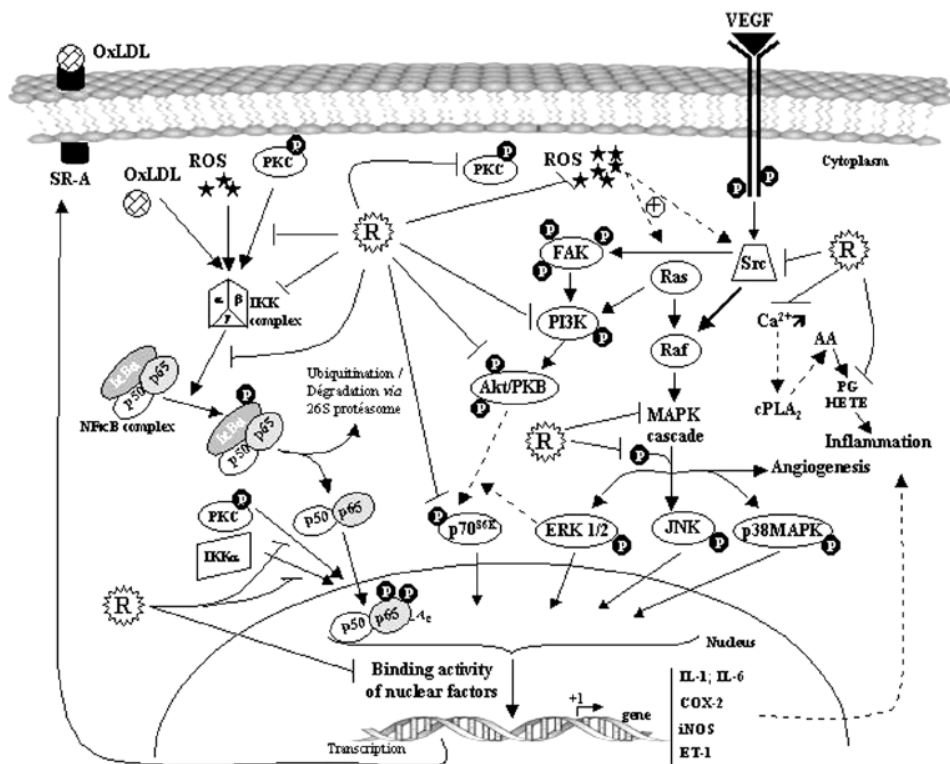


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