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PROSPECT

Multiple Role of Reactive Oxygen Species in the Arterial Wall

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Abstract Increased oxidative stress plays an important role in vascular dysfunction and atherogenesis. Both systemic factors, such as hypercholesterolemia and hyperglycemia, and local factors, such as activation of macrophages and T cells, may contribute to oxidative stress. Oxidation of lipids in lipoproteins and cell membranes leads to functionally important modifications of proteins that affect their recognition by cell surface receptors and protein–protein interactions within the cell, including DNA binding. Oxidized LDL and extracellular oxidation modulate oxidation-sensitive signaling pathways, but it is not clear to what extent this results from receptor-mediated activation or from direct effects on the intracellular redox-balance. Extensive evidence indicates that reactive oxygen species (ROS) regulate gene expression by modulating a large number of transcription factors, including the nuclear transcription factor kappa B (NFκB), the peroxisome proliferator activated receptorγ (PPARγ), and pathways linked to apoptosis. It is also increasingly recognized that cell differentiation and proliferation, cytokine expression, and programmed cell death are determined by the interactions between oxidation-sensitive regulatory pathways previously thought to lead to distinct outcomes. Because hypercholesterolemia exerts pro-oxidant effects both intra- and extracellularly and because increased ROS formation affects vascular reactivity and atherogenesis by modulating multiple signaling pathways and transcriptional events, future investigations of its atherogenic mechanisms should place greater emphasis on the net effect of such modulation on the expression of a large spectrum of genes. One way of doing this will be by defining clusters of genes responding to hypercholesterolemic stimuli — or interventions with structurally unrelated antioxidants — in analogous ways, irrespective of what regulatory pathway they are controlled by. Microarray technologies that allow simultaneous assessment of large numbers of genes may provide a tool for this approach. J. Cell. Biochem. 82: 674–682, 2001.

Key words: reactive oxygen species; transcription; atherosclerosis; NFκB; PPARγ; apoptosis; lipid oxidation

Reactive oxygen species (ROS) play an important role in many cardiovascular pathologies involving inflammatory processes, including atherosclerosis. Several pathogenic effects of increased ROS production have been identified. These include the oxidation of core lipids of lipoproteins and cell membranes which then modify apolipoproteins and other proteins, leading to their recognition by scavenger receptors. Similar modifications also influence DNA binding of regulatory proteins in the nucleus. It is also increasingly recognized that many cellular signaling pathways are oxidation-sensitive, and that ROS may provide a common link between pathways previously considered mainly from the perspective of their distinct outcomes, such as apoptosis or inflammation. A corollary of this is that pathogenic processes or interventions that influence ROS generation affect cell proliferation, differentiation, activity, and death by the balance of multiple effects on gene expression in individual cell types. This scenario is particularly complex in the arterial wall where continuous interactions between different arterial cell types and between the arterial wall and cellular and plasmatic components of the blood contribute to vascular dysfunction and atherogenesis.

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REACTIVE OXYGEN SPECIES AND OXIDIZED LDL IN THE ARTERIAL WALL

Numerous pathological conditions are associated with increased production of ROS in tissues and, to a lesser extent, in plasma

[Goldhaber and Weiss, 1992; Darley-Usmar and Halliwell, 1996; Griendling et al., 2000]. ROS in turn may be responsible for many aspects of vascular dysfunction and atherogenesis by modulating the expression of genes that influence vasotonus, recruitment of circulating cells into the arterial intima, cell proliferation, and apoptosis. Exposure of cells to ROS induces a local inflammatory response and the release of cytokines and growth factors, such as tumor necrosis factor α, interleukin1β, and interferonγ. These may then provide a positive feedback mechanism, for example by stimulating ROS-producing enzymes. Although cells are normally well protected from ROS by antioxidant defenses, including oxygen-radical scavenger enzymes, the rate of ROS formation can exceed the local antioxidant defense capacity and thus induce oxidant stress in arterial cells. More details on the source and effects of ROS in the artery wall are provided in Table I.

Oxidized LDL (OxLDL) is a prominent component of atherosclerotic lesions [Palinski et al., 1989] and is thought to promote atherogenesis by a variety of mechanisms [Steinberg, 1997]. One of these is the transformation of macrophages into foam cells that results from the uptake of large amounts of OxLDL via scavenger receptors. Both the uptake of oxidized proteins or phospholipids via scavenger receptors and interactions of macrophages with T cells and other vascular cells through cytokines can also lead to increased macrophage activation and generation of ROS. This may in turn promote the oxidation of LDL. Systemic factors such as hypercholesterolemia or diabetic hyperglycemia also contribute to oxidative stress in the arterial wall [Hunt et al., 1990; Chait et al., 1993; Lyons, 1993; Kawamura et al., 1994;Yan et al., 1994; Napoli et al., 1995, 1997a; Palinski et al., 1995; Reilly et al., 1998]. However, it is increasingly recognized that many atherogenic effects of OxLDL are exerted by modulation of gene expression in endothelial and other arterial cells, often by the same pathways that are affected by intracellular ROS. For example, extensive cell culture evidence indicates that exposure of cells to oxidized LDL affects NFκB-dependent gene expression and apoptotic signaling pathways in an oxygen-radical dependent way. The oxidation of LDL thus may be both a consequence of increased general or local oxidative stress and a cause of increased intracellular oxidative stress. Binding to specific cell surface receptors is clearly one mechanism by which this may influence activation of selected transcription factors, but we do not know to what extent extracellular oxidant stress may also directly influence the intracellular redox balance. Enhanced extracellular oxidation could for example deplete intracellular antioxidant defenses.

TRANSCRIPTION FACTORS AND CYTOKINES AFFECTED BY ROS

The consequences of enhanced oxidative stress in arterial cells are well documented in vitro and are generally consistent with in the

TABLE I. Reactive Oxygen Species in the Arterial Wall

1. Superoxide, singlet oxygen, hydrogen peroxide, and hydroxyl radical are most important [Harrison and Ohara, 1995: Am J Cardiol 75:75B–81B].
2. ROS may be generated by NAD(P)H oxidase [Berliner and Heinecke, 1996: Free Rad Biol Med 20:707–727; Griendling et al., 2000: Circ Res 86:494–501], xanthine oxidase [Wolin, 1996: Microcirculation 3:1–17; Cardillo et al., 1997: Hypertension 30:57–63], endothelial nitric oxide synthase [Ignarro et al., 1999: J Cardiovasc Pharmacol 34:879–886], cyclooxygenase [Ylä-Herttuala et al., 1991: J Clin Invest 87:1146–1152], myeloperoxidase [Berliner and Heinecke, 1996: Free Rad Biol Med 20:707–727], and lipoxygenase [Kunsch and Medford, 1999: Circ Res 85:753–766].
3. Cellular sources of ROS include blood-borne phagocytic cells, monocytes in the process of infiltrating the endothelial layer, as well as vascular smooth muscle cells, endothelial cells, and fibroblasts [Griendling and Alexander, 1997: Circulation 96:3264–3265].
4. Increased macrophage activation and generation of ROS can result from uptake of oxidized proteins or phospholipids of low-density lipoproteins via scavenger receptors or uptake of pathogens via phagocytosis, and from interactions of macrophages with T cells and other vascular cells through cytokines [Steinberg, 1997: J Biol Chem 272:20963–20966].
5. Arterial cells are normally well protected from ROS by antioxidant defenses, e.g., the oxygen-radical scavenger enzymes catalase, superoxide dismutase, and glutathione peroxidase, but the rate of ROS formation can exceed the local antioxidant defense capacity, resulting in increased oxidant stress [Kunsch and Medford, 1999: Circ Res 85:753–766].
6. ROS induce local inflammatory response and the release of cytokines and growth factors, such as tumor necrosis factor α, interleukin 1β, and interferon γ [Ross, 1993: Nature 362: 801–809; Ross, 1999: N Eng J Med 340:115–126].
7. ROS may affect both vascular dysfunction and atherogenesis [Kojda and Harrison, 1999: Cardiovasc Res 43:562–571; de Nigris et al., 2000: Biochem Pharmacol 59:1477–1487; de Nigris et al., 2000: Circulation 102:2111–2117].
8. Evidence for the importance of intra- and extracellular ROS in regulating gene expression is provided by inhibitory effects of antioxidants and oxygen-radical scavengers [Marui et al., 1993: J Clin Invest 92:1866–1874; Faruqi et al., 1994: J Clin Invest 94:592–600; Devaraj et al., 1996: J Clin Invest 98:756–763; Kunsch and Medford, 1999: Circ Res 85:753–766; de Nigris et al., 2000: Biochem Pharmacol 59:1477–1487; de Nigris et al., 2000: Circulation 102:2111–2117; Napoli et al., 2000: FASEB J 14: 1996–2007].

observation of cell migration and proliferation, the expression of pro-inflammatory cytokines and growth factors and the modification of the extracellular matrix observed in atherosclerotic lesions [Ross, 1999]. A large number of oxidation-sensitive transcription factors have also been identified [Marshall et al., 2000]. In the following, we will focus on the role of ROS in regulating the expression of genes via the nuclear transcription factor kappa B (NFκB), the peroxisome proliferator activated receptor γ (PPARγ), and the complex cascade of signaling events related to apoptosis, and discuss the potential interactions for atherosclerosis and vascular reactivity. Because in many of these studies, various forms of oxidized LDL and a variety of oxygen radical generating systems have been used, we will not differentiate between oxidative stress mediated via cell surface receptors and effects of enhanced intracellular ROS generation.

One of the earliest transcription factors recognized to be redox-sensitive is NFκB [Mercurio and Manning, 1999]. It is now well documented that exposure of cells to minimally oxidized LDL and specific oxidized phospholipids leads to upregulation of adhesion molecules and growth factors [Rajavashisth et al., 1990; Liao et al., 1993, 1994; Khan et al., 1995; Watson et al., 1997] and to vascular dysfunction [Napoli et al., 1997b]. These include adhesion molecules VCAM-1 and ICAM-1, monocyte chemotactic protein 1, nitric oxide synthase 2, and several proinflammatory cytokines, e.g., tumor necrosis factor α and interleukin 2. Cytoplasmic NFκB is activated by the cleavage of IκB from the p50–65 heterodimer, which is then translocated to the nucleus. Cleavage of IκB requires an oxidizing milieu and appears to be one of the mechanisms through which ROS activate NFκB [Hamilton et al., 1998]. Another is through Ras [Lander et al., 1995]. Surprisingly, in the nucleus binding of the p50 subunit of the active NFκB dimer to DNA occurs at basic pH and is inhibited by ROS [Matthews et al., 1993]. The importance of the cytosolic action of ROS is indicated by the observation that antioxidants prevent the upregulation of VCAM-1 and monocyte adhesion in vitro [Erl et al., 1997] and in vivo [Fruebis et al., 1997, 1999]. However, the effects of antioxidant treatment on disease outcome are anything but predictable on the basis of in vitro effects of antioxidants on selected pathways [Stephens et al., 1996; Steinberg, 1997; Pryor 2000; Yusuf et al., 2000].

Studies in cultured endothelial and smooth muscle cells demonstrated that multiple apoptotic signaling pathways are also influenced by ROS [Dimmeler et al., 1997; Kinscherf et al., 1998; Li et al., 1998; Sata and Walsh, 1998a, b; Napoli et al., 2000a]. Even mildly oxidized LDL likely to be found in early atherosclerotic lesions activates both FasL and TNF receptor pathways, increases proapoptotic, and decreases antiapoptotic proteins of the Bcl-2 family, and results in a marked activation of Class I and II caspases [Napoli et al., 2000a], the presumed principal end-effectors of apoptosis. Mildly oxidized LDL generated by the xanthine/xanthine oxidase system also leads to activation of the mitogen-activated protein kinase (MAPK) and Jun kinase (JNK) pathways [de Nigris et al., 2000a; Napoli et al., 2000a]. The role of ROS in this process is highlighted by the marked reduction of this activation by the presence of oxygen radical scavengers during LDL oxidation. Several other redox-sensitive transcription factors are similarly affected by oxidized LDL and scavengers. These include ATF-2, ELK-1, CREB, NFkB, AP-1, p53, and the c-Myc/Max complex and its binding factors, E2F and AP-2 [de Nigris et al., 2000a, b; Napoli et al., 2000a]. Some of these factors have been linked to apoptosis, such as p53 [Chen et al., 1996], whereas others appear to promote cell differentiation and proliferation, e.g., AP-1 [Marshall et al., 2000] and NFκB [Mercurio and Manning, 1999]. The upregulation of some of the above factors may simply be due to the activation of MAP and Jun kinases by ROS and/or peroxidative end-products, and their importance for cell survival has yet to be established. Nevertheless, it appears that activation of "apoptotic" signaling is accompanied by the activation of growth-promoting transcription factors, which may constitute a compensatory mechanism to limit cell death. Indeed, the extent of actual cell death observed in atherosclerotic lesions is much smaller than what would be assumed on the basis of the ubiquitous presence of OxLDL in atherosclerotic lesions, its powerful pro-apoptotic signaling effects, and the high percentage of TdT-mediated dUTP nick-end labeling (TUNEL) positive cells detected in lesions [Kockx and Herman, 2000]. It therefore is likely that the biological outcome, in terms of cell survival and activity, is the result of

multiple potentially antagonistic actions of ROS on different pathways, as well as of other activators influencing individual pathways.

A third example of an oxidation-sensitive pathway is PPARγ. PPARγ is a nuclear receptor that regulates fat cell development and glucose homeostasis, and is the molecular target of insulin-sensitizing agents used for the management of type 2 diabetes mellitus [Willson et al., 2000]. PPARγ is highly expressed in macrophage/foam cells of atherosclerotic lesions [Ricote et al., 1998a; Tontonoz et al., 1998]. In vitro, ligand-induced PPARγ activation upregulates the scavenger receptor CD36, but downregulates proinflammatory genes, including TNFα, IL-1β, inducible NO synthase (iNOS) and gelatinase B [Jiang et al., 1998; Marx et al., 1998; Nagy et al., 1998; Ricote et al., 1998b; Tontonoz et al., 1998], as well as the expression of the MCP-1 receptor, CCR2 [Han et al., 2000] and leukocyte-endothelial cell interaction [Jackson et al., 1999; Pasceri et al., 2000]. Several groups, including ours, have reported a striking co-localization of PPARγ and OxLDL in human and murine lesions [Nagy et al., 1998; Ricote et al., 1998a], and in vitro experiments confirmed that OxLDL upregulates the expression of PPARγ [Ricote et al., 1998a; Huang et al., 1999]. One could therefore postulate that PPARγ activation in vivo would be antiatherogenic, by downregulating the expression of pro-inflammatory cytokines, or pro-atherogenic, by promoting macrophage expression of the scavenger receptor CD36 and ensuing foam cell formation. An initial in vivo study in LDL receptor-deficient mice with high doses of two distinct synthetic PPARγ activators indicated the prevalence of the antiatherogenic effect [Li et al., 2000]. However, given other effects of these PPAR-ligands, in particular the activation of the ATP-binding cassette transporter gene A1 (ABC-A1) [Chawla et al., 2001], the impact of the downregulation of inflammatory genes and upregulation of CD36 on atherosclerosis remains to be determined.

Enhanced oxidative stress is not only important in atherosclerosis, but also in vascular dysfunction. Altered vasomotor regulation plays a central pathophysiological role in vascular diseases and is partially reversed by antioxidant treatment. Hypertension may promote atherogenesis by activating the renin-angiotensin system, stimulating NADPH oxidase [Rajagopalan et al., 1996], and increasing expression of pro-inflammatory genes in the vessel wall [Chen et al., 1998]. However, shear stress per se can also trigger oxidation-sensitive gene expression [Ishizaka et al., 1997; De Keulenaer et al., 1998]. OxLDL exerts profound effects on the vasomotor response of isolated arteries to various stimuli that closely mimic the vascular dysfunction associated with hypercholesterolemia and atherosclerosis in humans [Cox and Cohen, 1996]. OxLDL antagonizes the vasodilatory effect of NO, and ROS and NO antagonize each other at the level of intracellular signaling. For example, NO inhibits cleavage of IkB and NFkB activation that is enhanced by ROS [Marshall et al., 2000]. NO and NFκB signaling pathways are intimately linked, and NFκB activation is essential for the activation of the gene encoding NO synthase 2 (NOS2) [Xie et al., 1994]. Diabetes, which promotes the generation of superoxide anions and LDL oxidation, activates oxidation-sensitive pathways as well [Pieper and Riazul, 1997]. Hyperglycemia also decreases the bioavailability of NO and induces endothelial dysfunction [Bucala et al., 1991; Kakoki et al., 1999; Posch et al., 1999], either through ROS, OxLDL, or advanced glycation end products (AGE), which are recognized by cell surface receptors [Schmidt et al., 1995].

Given the sheer number of pathways affected by ROS and the likelihood for potential interactions and compensatory mechanisms, it is evident that identifying oxidation-sensitive pathways and genes that significantly contribute to or inhibit atherosclerosis is difficult. The conventional approach would be to determine the effect of prooxidant stimuli or OxLDL on specific pathways, and to predict its effects on atherosclerosis on the basis of the effects of individual genes modulated by this pathway. However, establishing a causal role remains beyond reach even for genes for which viable knockout models have clearly shown an atherogenic role, simply because so many other genes may be affected simultaneously. Figure 1 provides a schematic overview of some of the difficulties encountered in establishing the mechanisms by which lipid peroxidation and ROS formation modulate atherogenesis.

To determine the role of increased oxidation or interventions modulating it, a different approach appears more promising. Recent progress in microarray techniques makes it possible to assesses the mRNA expression of

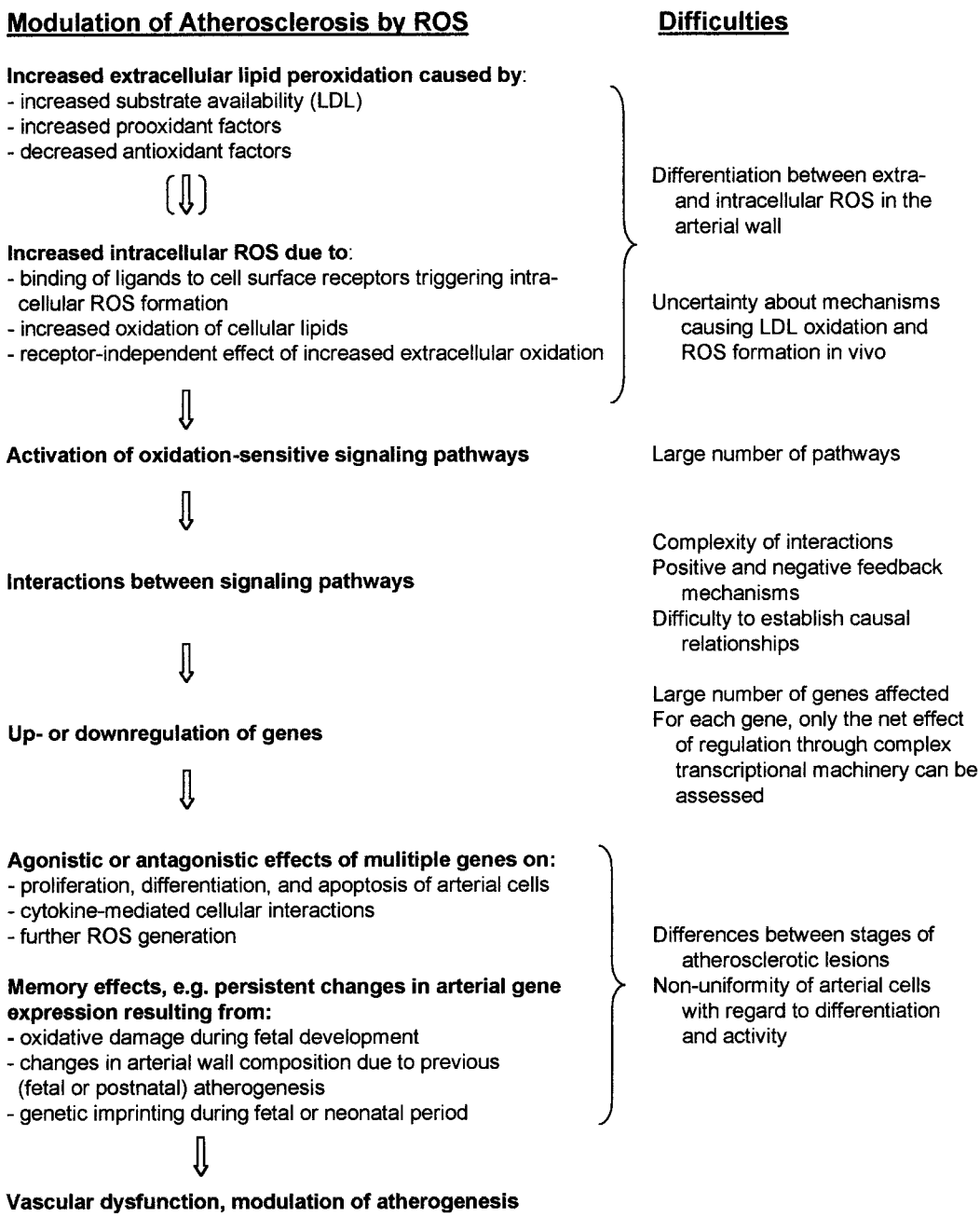

Fig. 1. Role of ROS in atherogenesis and main experimental difficulties encountered in determining the mechanisms by which lipid peroxidation and ROS modulate atherogenesis, using conventional approaches. Simultaneous assessment of the expression of a broad spectrum of genes by array techniques and correlation of expression patterns with the effect on atherosclerosis may contribute to overcome these problems.

thousands of genes at a time [Bowtell 1999; Gerhold et al., 1999]. Gene chips for 36,000 murine genes and expressed sequence tags (ESTs) are commercially available and the technology to generate custom arrays comprising several hundred genes is also beginning to spread. This offers a way to determine a large spectrum of changes associated with increased oxidative stress at once and to identify, by appropriate data mining strategies [Eisen et al., 1998], clusters of genes regulated in analogous ways, irrespective of the pathway determining their expression. Such clusters can then be correlated with atherogenesis, verified under

different experimental conditions, and used to identify surrogate parameters that predict the progression or regression of the disease. Fortunately, mice have become a favorite model for atherosclerosis research [reviewed in Palinski et al., 2000].

An example of how the approach proposed above could be used to answer important questions is our research on the fetal origins of atherosclerosis. It has recently been recognized that the onset of atherogenesis may occur as early as during fetal development, and that maternal hypercholesterolemia during pregnancy is associated with enhanced oxidation and markedly increased sizes of early fatty streaks (the earliest stage of atherosclerotic lesions) in the fetal aorta of humans [Napoli et al., 1997c; Napoli et al., 1999a]. Maternal hypercholesterolemia and enhanced fetal lesion formation is associated with a much faster progression of atherosclerosis in normocholesterolemic children [Napoli et al., 1999b], suggesting that pathogenic events in the fetal aorta persist over time and influence the susceptibility to atherogenesis later in life. Experiments in genetically homogeneous animal models demonstrated a causal role of oxidation in fetal lesion formation and the partial reversibility by maternal treatment with vitamin E [Napoli et al., 2000b]. We therefore postulate that oxidation-sensitive signaling is affected in fetal arteries, similar to that described above, and that this leads to persistent changes in gene expression or persistent compositional differences of the arterial wall which render it more susceptible to atherogenesis [Palinski and Napoli, 1999; Napoli and Palinski, 2001]. Examples of permanent effects caused by temporary exposure to hypercholesterolemia during the neonatal period have previously been reported [Li et al., 1980], and the mechanisms of parental imprinting during certain developmental stages are also increasingly understood [Villar and Pedersen, 1994], but it is unknown whether the consequences of fetal lesion formation stem from such mechanisms, whether they reflect the persistence over time of subtle compositional changes in the arterial wall that account for changes in overall gene expression, or whether altered gene expression persists in individual cells.

Gene chip experiments currently under way in our laboratory will establish whether maternal hypercholesterolemia or pathogenic effects occurring during fetal development result in persistent changes in arterial gene expression, long after birth and in the absence of acute stimulation. Obviously, applying microarray techniques to the arterial wall is complicated by the cellular heterogeneity and lack of synchronization of cell cycles. This may be overcome, either by focusing on the normal arterial wall prior to the onset of intimal thickening, or by selectively extracting specific cells from defined stages of atherosclerotic lesions by laser capture microscopy [Bonner et al., 1997]. If these studies can demonstrate that fetal pathogenic effects persist later in life and affect the susceptibility to atherogenesis in response to conventional risk factors, this may open the door to a novel preventive approach, i.e., treatment of hypercholesterolemic mothers during pregnancy.

In summary, increased ROS formation is a common consequence of many pathologies, including hypercholesterolemia and diabetes, and provides a link between signaling pathways and transcriptional events that regulate the expression of a large number of genes. These genes govern cellular recruitment, differentiation, proliferation, and death, and may profoundly modulate atherogenesis. The complexity of the interaction occurring both within the cell and between the cell and its environment makes it difficult to assign biological effects, such as the modulation of atherogenesis, to the modulation of individual pathways or genes. However, microarrays offer a promising new tool to study biologically relevant consequences of the complex interactions between pathways.

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