SIGNAL TRANSDUCTION
AND HUMAN DISEASE
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Edited by

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To my three muses
Beth, Kira and Nadia

TF

To my four pillars
Silvia, Sarah, Naomi, and Juanita

JSG
CONTENTS

Acknowledgments ix
Contributors xi
Introduction xv

1 Atherosclerosis: Signal Transduction by Oxygen and Nitrogen Radicals 1
Jonathan M. Hill, Ilisa I. Rovira, and Toren Finkel

2 NF-κB: A Key Signaling Pathway in Asthma 23
Stewart J. Levine

3 Molecular Mechanisms of Cancer 71
Akrit Sodhi, Silvia Montaner, and J. Silvio Gutkind

4 Apoptotic Pathways in Cancer Progression and Treatment 143
Joya Chandra and Scott H. Kaufmann

5 Molecular and Cellular Aspects of Insulin Resistance: Implications for Diabetes 171
Derek Le Roith, Michael J. Quon, and Yehiel Zick

6 Dysfunction of G Protein-Regulated Pathways and Endocrine Diseases 201
William F. Simonds

7 Bacterial Regulation of the Cytoskeleton 233
Jeremy W. Peck, Dora C. Stylianou, and Peter D. Burbelo

8 Bacterial Toxins and Diarrhea 259
Walter A. Patton, Joel Moss, and Martha Vaughan

9 Molecular Basis of Severe Combined Immunodeficiency: Lessons from Cytokine Signaling Pathways 279
Roberta Visconti, Fabio Candotti, and John J. O'Shea
10 Mast Cell-Related Diseases: Genetics, Signaling Pathways, and Novel Therapies 307
Michael A. Beaven and Thomas R. Hundley

11 Rheumatology and Signal Transduction 357
Keith M. Hull and Daniel L. Kastner

12 Molecular Mechanisms of Neurodegenerative Disorders 377
Benjamin Wolozin

13 Neurotrophic Signaling in Mood Disorders 411
Jing Du, Todd D. Gould, and Husseini K. Manji

14 Inhibiting Signaling Pathways Through Rational Drug Design 447
James N. Topper and Neill A. Giese

Index 459
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INTRODUCTION

Flower in the crannied wall,
I pluck you out of the crannies,
I hold you here, root and all, in my hand,
Little flower—but if I could understand
What you are, root and all, and all in all,
I should know what God and man is.

From “Flower in the Crannied Wall”
Alfred Lord Tennyson
1809–1892

Pick up any newspaper or turn on any television set, and undoubtedly you will be confronted by the dizzying array and breathtaking speed of scientific and medical advances. Future historians will certainly note that a mere 50 years separated the initial discovery of the structure of DNA from the description of the complete sequence of the human genome. Similarly, the pace of scientific discovery has forever altered our expectations and perspectives. For instance, in the past, deciphering the causative mutations for conditions such as sickle cell anemia or familiar hypercholesterolemia would take years of meticulous planning and painstaking work and, in the end, the isolation of the culpable gene would shake the very foundation of science and medicine. In contrast, these days the genetic bases for diseases are reported with such frequency that their discovery is often treated with the indifference one reserves for stories on insurance premiums or crop forecasts.

Despite the pace of medical research, the sad fact remains, however, that the incidence of many fatal diseases continues to increase. In addition, although new treatments are continually discovered and tested, it is also safe to say that today the life expectancy following the diagnosis of an advanced solid tumor or end-stage congestive heart failure remains exceedingly short. What then is the impact of our increasing knowledge of human biology on our ability to treat the most severe and crippling of human diseases? The short answer is that although it is too early to know for sure, certain promising signs are emerging. Indeed, there appears to be a growing list of drugs being tested in early clinical trials that translate insight garnered from basic laboratory research to specifically target molecular pathways fueling disease. For example, once-fatal leukemias can now be successfully treated with drugs such as the newly described agent Gleevec, which inhibits a kinase specifically activated in the process of malignant transformation. Similarly, other novel agents that target receptor tyrosine kinases, such as the epidermal growth factor (EGF) receptor, appear to be promising drugs to treat a number of solid tumors.

When surveying the field, we could find no text that straddled the productive interface between modern biology and modern medicine. Indeed, we
began to feel that a laboratory researcher working in the field of asthma might be very conversant with the intricate molecular signaling pathway of NF-κB and its myriad intervening components and target genes, yet he or she might never have been exposed to the simple clinical tool of flow-volume loops or seen graphically the effects of bronchodilators on airway resistance. Conversely, a rheumatologist might be quite adept at examining a joint and developing an appropriate differential diagnosis but be quite unaware of the details surrounding TNF signaling. In the first edition of this book, we have attempted to bring these two complementary approaches into one volume. Together with our contributors, we have labored to describe a host of disease processes from common conditions such as atherosclerosis and cancer to disorders such as TRAPS, a rare rheumatological syndrome characterized by periodic fevers and rashes. Within many of the chapters, where appropriate, we have first tried to give the reader a sense of the disease process, what it affects, how it presents, how common it is, and what the current treatments are. These clinical descriptions are not meant to be exhaustive but rather to serve as an outline to the reader regarding the disease’s manifestations and current treatment options. After this introduction, we usually present a more in depth discussion of one or two signal transduction pathways or biological process relevant to the disease. Throughout these fourteen chapters we have endeavored to cover most of the major signaling pathways using a variety of different human diseases as our framework and point of embarkation.

The book is divided like many medical textbooks into subspecialty areas. In our case this includes sections in cardiopulmonary disease, oncology, endocrinology, infectious disease, allergy/rheumatology, and neurology/psychiatry. Diseases discussed include among others cancer, asthma, atherosclerosis, diabetes, rheumatoid arthritis, Parkinson disease, and depression. In addition, we outline the current understanding of diverse pathways from MAPK activation in cancer to the role of NF-κB in asthma and arthritis, from JAK/STAT signaling in immune deficiencies to the molecular basis of dysentery.

We begin with cardiology, discussing the basis of atherosclerosis and the role that small diffusible radical species such as nitric oxide and superoxide have on the vessel wall. Rather than viewing them simply as toxic molecules, we show that these reactive oxygen species play an important role in vascular homeostasis. Pharmacological manipulation of these pathways has in fact been known for a century or more, as nitroglycerin (a nitric oxide generator) has been widely used by symptomatic patients for treating chest pain (i.e., angina pectoris). Indeed, Alfred Noble, the Swedish benefactor of the Noble Prizes, used nitroglycerin as a starting point for his discovery of dynamite in the 1860s. Close to 150 years later, three scientists would share a Nobel Prize for the understanding that nitric oxide regulates vascular tone, with pharmacological agents such as nitroglycerin deriving their clinical benefit by mimicking these effects. The further description of other agents such as Viagra, which prolong the half-life of nitric oxide in certain, shall we say, critical organs, have reenforced the importance of this pathway in health and disease.

After this description of atherosclerosis we discuss the growing epidemic of asthma, a disease that affects both children and adults. We use this condition to discuss an essential regulator of the inflammatory process, namely, the NF-κB pathway. In particular, we discuss activation of the NF-κB pathway through cytokine receptors such as the tumor necrosis factor (TNF) receptor.
We next delve into cancer biology. One chapter in this section is a general review of the molecular mechanisms of cancer, focusing on the key biochemical pathways involved in cell cycle regulation and the acquisition of the malignant phenotype. Among the areas discussed are the Ras-MAPK pathway, small GTPases and exchange factors, p53, Rb, and other tumor suppressors, as well as receptor tyrosine kinases. After this discussion, we discuss in a separate chapter the biology of programmed cell death including caspases, the Bcl-2 family of pro- and antiapoptotic proteins, and the Akt kinase. Our goal is to demonstrate how these pathways and their intricate interplay relate to tumor progression and ultimately how they will shape future treatment modalities.

We next move into the area of endocrinology with two separate chapters. The first chapter deals with the molecular basis for diabetes. This chapter primarily discusses the basis for insulin resistance and discusses downstream signaling from the insulin receptor and other relevant receptor tyrosine kinases. The following chapter deals with G protein-coupled receptors (GPCRs) and in particular the multiple endocrine manifestations resulting from inappropriate GPCR activity.

After the section on endocrinology we move on to infectious diseases. The first chapter discusses the interaction of bacteria with the cell and in particular the lessons these interactions have taught us regarding dynamic regulation of the cytoskeleton. The next chapter deals with the molecular basis underlying the diarrhea associated with infectious agents such as cholera or *Escherichia coli* that result in a staggering amount of mortality each year in the developing world. The toxins from these organisms have provided a number of valuable lessons in cell biology, and the authors provide an in-depth description of an interesting posttranslational modification, ADP-ribosylation.

The next chapters are concerned with allergy and rheumatology. We begin with a primer on severe combined immunodeficiencies, a constellation of over 95 different syndromes that impact the immune system. This syndrome is a natural starting point to discuss the world of cytokine signaling and the downstream pathway regulated by JAK and STAT proteins. We next discuss the basis for allergic reaction, from the devastating forms of anaphylaxis to milder syndromes such as hay fever, paying particular attention to the mast cell as the underlying cell type responsible for these allergic responses. Finally, we discuss two rheumatological conditions, the rare periodic fever syndrome TRAPS and the more common rheumatoid arthritis. These two syndromes allow for a discussion of TNF signaling and a look at NF-κB signaling in another disease context.

In the last major section we turn to the brain to discuss both neurological diseases and mood disturbances. In the first chapter, we discuss a variety of debilitating diseases characterized histologically by neurological degeneration. This section allows for a discussion of protein aggregation and the various intracellular processes stimulated by pathological protein aggregates. In the next-to-last chapter we discuss syndromes such as depression and bipolar disease. These disorders, which can in their severe form be life threatening, provide the impetus to discuss signaling through the neurotrophic receptors and the regulation of the CREB transcription factor. The last chapter is devoted to novel drug development and in particular how one goes from candidate target to candidate drug, in essence, how one translates the emerging knowledge of the basic scientific advance into a practical and useful medicine.
As you can see from these brief descriptions, we have, for the benefit of clarity, limited each section to covering only a handful of relevant pathways. Clearly, for instance, the MAPK pathway affects a host of diseases besides cancer and would be just as relevant to talk about in the context of diabetes or a number of neurological conditions. Similar arguments could undoubtedly be made for other signaling pathways such as NF-κB or nitric oxide that have important manifestations in a number of diseases. Therefore, the reader is cautioned that these fourteen chapters are meant as an overview and guide for future explorations. Although we have chosen to discuss important pathways for disease initiation or progression, signal transduction is an integrated subject and no single pathway can or should be viewed in total isolation.

The worlds of laboratory science and clinical medicine are both moving at breakneck speed. As they grow, the tools, techniques, and language of these two areas invariably become more specialized and unique to each discipline. We hope that we have managed in this volume to provide the reader a footing in both camps, in essence, to provide both a big picture as well as giving a sense of the individual brush strokes. We believe that this holistic approach will allow the reader to conveniently integrate both the important clinical and molecular aspects of a number of important disease processes. Such a range of knowledge will undoubtedly be essential if we are to be successful in creating the next generation of molecular therapies.
CHAPTER 1

ATHEROSCLEROSIS: SIGNAL TRANSDUCTION BY OXYGEN AND NITROGEN RADICALS

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INTRODUCTION

We are faced with a growing pandemic of cardiovascular disease and stroke at the start of the third millennium. According to World Health Organization estimates, in 1999, cardiovascular disease contributed to one-third of all deaths, with 78% of those deaths occurring in low- and middle-income countries. Atherosclerosis, a disease affecting large arteries, is the underlying cause of most of these deaths. In developed societies, despite access to complex drug therapy and invasive treatment, it remains the number one killer, contributing to nearly one-half of all deaths, while in the developing world, economic transition and industrialization appear to be bringing about lifestyle changes destined to create a new generation of cardiovascular disease victims. Indeed, by 2010 it is estimated that in the developing world, cardiovascular disease will be the leading cause of death. The majority of this mortality burden appears to be at least partly preventable and controllable.

Although there were early descriptions of atherosclerosis in Egyptian mummies, the first careful anatomic and physiological descriptions of atherosclerosis date from the mid-eighteenth century. In recent years, a number of important studies have allowed for a more fundamental understanding of disease mechanisms, with some of these studies providing the first dissection of the relevant intracellular signaling pathways. These studies have pointed the way for the development of new pharmacologic therapies and novel risk reduction strategies. In this chapter, we outline the epidemiological and clinical aspects of atherosclerotic disease from the early stages of endothelial dysfunction and plaque for-
mation to eventual plaque rupture. In an overview of signal transduction mechanisms in atherosclerosis, we focus on just one aspect of the disease process by describing the biology of nitric oxide and other reactive oxygen species (ROS) in the arterial wall. In a review of modern treatment approaches we underscore how the understanding of signaling pathways has led to better therapeutic options.

**ATHEROSCLEROTIC LESION DEVELOPMENT AND CLINICAL PRESENTATIONS**

Large arteries are comprised of three distinct layers. The intima is the endoluminal layer and is lined with endothelial cells bound to a sheet of connective tissue made up predominantly of collagen and proteoglycans. It is in this layer that many of the initial and predominant changes of atherosclerosis occur. The media consists of smooth muscle cells, whereas the adventitia consists mostly of connective tissue elements such as fibroblasts. In general, it is the outermost endothelial layer and the underlying smooth muscle cell layer (i.e., the intima and media) that are thought to be the most important in maintaining overall vascular tone.

In normal individuals, physiological increases in blood flow are caused in large part by endothelium-mediated vasodilatation. This enables blood flow to increase in line with tissue oxygen demands. The major endothelium-derived relaxing factor (EDRE) was discovered by Furchott and Zawadzki (Furchgott and Zawadzki, 1980) and was subsequently identified chemically as nitric oxide (NO) (Palmer et al., 1987; Ignarro et al., 1986; Ignarro et al., 1987). In addition to NO there are a number of other vasodilator and vasoconstrictor substances that regulate vascular tone and homeostasis including endothelin-1, prostacyclin, prostaglandin H2, and the endothelium-derived hyperpolarizing factor EDHF. In the coronary circulation there is evidence that NO is constantly released from the endothelium (Quyyumi et al., 1995) to maintain a basal state of vasodilatation and to counteract the vasoconstricting effects of substances such as noradrenalin, angiotensin, and endothelin.

Clinical assessment of the endothelial function of the coronary and peripheral circulations can be measured by monitoring the vasodilator response to endothelium-dependent agonists such as acetylcholine. Dysfunctional endothelium is characterized by reduced vasodilatation in response to agents such as acetylcholine. It should be noted that acetylcholine, besides stimulating NO release, also stimulates release of other vasodilating substances such as EDHF. More recently, an ultrasound technique measuring brachial artery flow-mediated vasodilatation allows the repetitive and noninvasive measurement of endothelial function in human subjects (Celermajer et al., 1992).

The Framingham Study (Stokes et al., 1987; Kannel, 2000; D’Agostino et al., 2000) is probably the best-known large-scale epidemiological study
that generated the idea of specific “risk factors.” Data began emerging from this study in the 1960s showing the relative contributions of multiple risk factors to the pathogenesis of atherosclerosis and its numerous clinical manifestations. They can be divided into factors with a predominant genetic component and those that are largely environmental (Table 1.1). Individual risk factors can interact with each other and may synergistically affect the progression of the disease. Data from the original Framingham Study were extremely important in the identification of a number of classic risk factors such as smoking, diabetes, and hypertension. Recently, in addition to these conventional risk factors, there are a number of emerging novel atherosclerotic risk factors such as homocysteine levels, fibrinogen levels, and potentially infectious agents.

The most common way for atherosclerotic disease to present clinically is the development of angina. This is experienced by patients as a tightness or pain across the chest and sometimes down the arm. It is the result of a narrowing in a coronary artery supplying the heart muscle, reducing the blood flow and causing myocardial ischemia (Fig. 1.1). Anginal symptoms may be precipitated by situations requiring increased myocardial blood flow, such as during exercise, anxiety, and cold weather and after heavy meals. They are often associated with a feeling of breathlessness. Atherosclerotic disease affecting the peripheral arteries presents in the same way when the narrowed arteries cannot supply enough blood to meet the tissue oxygen demands. The symptoms for patients with peripheral vascular disease is often described as a tightness or aching in the calf muscles after exercise. This syndrome is called intermittent claudication. As with most symptoms associated with vascular

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TABLE 1.1. Established and Emerging Risk Factors for Coronary Artery Disease

<table>
<thead>
<tr>
<th>Risk Factors with Genetic Component</th>
<th>Risk Factors Triggered by the Environment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid abnormalities</td>
<td>High-fat diet</td>
</tr>
<tr>
<td>$\uparrow$LDL/VLDL, $\downarrow$HDL, or $\uparrow$Lp(a)</td>
<td>Smoking</td>
</tr>
<tr>
<td>Abnormal hemostatic factors</td>
<td>Lack of exercise</td>
</tr>
<tr>
<td>$\uparrow$Fibrinogen, $\uparrow$PAI-1</td>
<td>Infection (e.g., <em>Chlamydia pneumoniae</em>, CMV)</td>
</tr>
<tr>
<td>Depression</td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus and insulin resistance</td>
<td></td>
</tr>
<tr>
<td>Family history</td>
<td></td>
</tr>
<tr>
<td>Homocysteinemia</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
</tr>
<tr>
<td>Male gender</td>
<td></td>
</tr>
<tr>
<td>Obesity</td>
<td></td>
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</table>

Risk factors are divided into those that have some genetic basis and those that are purely environmental.
disease, the disease tends to be slowly progressive, reflecting the chronic nature of plaque progression.

The most common presentations of atherosclerosis in the acute setting are myocardial infarction, unstable angina, and stroke. The precipitating event is a result of the acute instability of an atherosclerotic plaque, the surface of which may rupture, causing acute thrombosis and vessel occlusion. Myocardial infarction produces irreversible necrosis of part of the heart muscle and is often fatal before the patient reaches the hospital. It can be treated with drugs targeting the thrombotic cascade and clot formation or by opening the closed artery with a small balloon (angioplasty). At present, little clinical information is available to guide patients or physicians as to when a plaque will convert from a stable lesion to the much more dangerous unstable plaque.

**REDOX SIGNALING PATHWAYS IN ATHEROSCLEROSIS**

The concept that endothelial injury is the initiating factor in atherosclerosis dates back to the observations of Virchow, who suggested that atherosclerosis developed after mechanical irritation to the intima, which in turn caused degenerative and inflammatory responses leading to local cellular proliferation (Virchow, 1856). It is now generally believed that, in addition to these mechanical forces, risk factors such as smoking, diabetes, and hypercholesterolemia function as continuous endothelial damaging agents. It is also thought by many, but certainly not
all investigators, that oxidative stress is the common mediator of a host of environmental and genetic cardiovascular risk factors (Fig. 1.2). As such, risk factors are thought to act in large part by promoting vascular oxidative stress. Because superoxide can readily inactivate NO, a rise in oxidative stress, particularly in superoxide levels, can counteract the biological activity of NO. Hence, an understanding of NO and other ROS is thought to be critical to understanding the initiation and progression of cardiovascular disease.

Consistent with their ability to induce oxidative stress, atherosclerotic risk factors appear to modulate normal physiological signal transduction pathways within the vessel wall to induce a syndrome termed “endothelial dysfunction.” For the purposes of this review, we define endothelial dysfunction as an impairment of endothelial vasodilator function principally related to the bioavailability of NO. This is most often detected as an impairment of the vascular response to agents such as acetylcholine. The presence of endothelial dysfunction even without overt macroscopic atherosclerotic disease was recently demonstrated to predict adverse cardiovascular events and long-term outcome (Schachinger et al., 2000). As such, the clinical syndrome of endothelial dysfunction may be one of the earliest markers of the atherogenic process.

Early pioneering experiments by Furchgott and colleagues determined that the ability of acetylcholine to induce vasorelaxation required a functional endothelial layer. Later experiments demonstrated that
Acetycholine induced the synthesis of NO. That a small, diffusible gas could be purposely produced within the vessel wall and have an important physiological role was a remarkable departure from conventional thinking with regards to all forms of ROS. The major discoverers of this concept, Furchgott, Ignarro, and Murad, would go on to share the Nobel Prize for Medicine or Physiology in 1998. The production of NO is now known to occur via the action of nitric oxide synthase (NOS), an enzyme family that catalyzes the conversion of L-arginine to L-citrulline in the presence of molecular oxygen and NADPH to yield NO. In addition to its principal role in stimulating vasorelaxation by the production of cGMP in smooth muscle cells, NO and its derivatives play a key role in the development of atherosclerosis by regulating monocyte and platelet adhesion, altering endothelial permeability, and inhibiting vascular smooth muscle cell proliferation and migration (Garg and Hassid, 1989; Cornwell et al., 1994).

There are three distinct isoforms of NOS, arising from three separate genes, with variations in their structure reflecting their specific in vivo functions (Stuehr, 1997). Each enzyme is a highly complex system with distinct functional domains and a multitude of cofactors and prosthetic groups. The enzyme generally functions as a homodimer of identical subunits each bearing two major functional domains: an N-terminal oxygenase, which binds heme and tetrahydrobiopterin (BH4) as well as the substrate L-arginine, and a C-terminal reductase, which contains the binding sites for NADPH, FAD, and FMN. The enzyme is similar to the cytochrome P-450 family of enzymes, especially in its ability to catalyze flavin-mediated electron transport from the electron donor NADPH to a prosthetic heme group. The calmodulin binding domain (CaM) lies between these two functional regions of NOS and is integral to structure and enzymatic function. In the absence of appropriate levels of the substrate L-arginine or BH4, NOS enzymes can produce superoxide and H₂O₂ (NOS uncoupling). The physiological role of this uncoupling is not completely understood, although some recent reports suggest that NOS-produced superoxide can also function as a signaling molecule (Wang et al., 2000).

The main endothelial isoform, eNOS (also called NOS3), differs from the other isoforms with a unique subcellular localization. This localization is achieved because only eNOS is acylated by both palmitate and myristate. Specific residues are modified with Cys-15 and Cys-26 undergoing palmitoylation while an N-terminal glycine undergoes myristoylation (Shaul et al., 1996). Although the myristoylation is an irreversible modification, the palmitoylation step is reversible and subject to physiological regulation by a host of agonists that increase intracellular calcium. One end result of this complex and unique posttranslational modification is that eNOS is not uniformly distributed throughout the endothelial cell membrane but is instead confined to plasmalemmal microdomains known as calveolae. These structures are becoming increasingly important in signal transduction and represent areas in which signaling proteins and their downstream effectors appear to be
A number of caveolin proteins have been defined, and it appears that eNOS can directly bind to both caveolin-1 and caveolin-3 (Feron et al., 1996), with binding appearing to inhibit NOS activity. Recent studies supporting the importance of these interactions come from mice with targeted deletions of caveolin-1, in which it has been observed that there is a major alteration in NO-mediated vasorelaxation (Drab et al., 2001).

Given that eNOS is the gene product responsible for producing the EDRF described by Furchgott, it is not surprising that a number of reports have examined the physiological regulation of the enzyme at both the transcriptional and posttranslational levels. These studies demonstrated that a number of important physiological stimuli regulate eNOS gene expression, such as shear stress, oxidized LDL, and exercise training (Uematsu et al., 1995; Sessa et al., 1994). Some reports suggested that at early stages of atherosclerotic lesion development eNOS expression may be downregulated through a decrease in transcription and a destabilization of mRNA, whereas as the lesion matures the overall level of eNOS expression may actually increase. The physiological significance of these observations is unclear. In addition, there is not always a clear relationship between mRNA level, protein levels, and enzymatic activity, suggesting the possibility of additional layers of complexity and regulation by yet undefined posttranscriptional and posttranslational mechanisms.

Another emerging important form of regulation of eNOS activity appears to be protein phosphorylation. The enzyme has a number of consensus sequence sites for phosphorylation by protein kinase A (PKA), protein kinase B (Akt), protein kinase C (PKC), and calmodulin kinase II. There is now evidence that in addition to the phosphorylation of serine residues (Michel et al., 1993) eNOS can also be tyrosine phosphorylated (Garcia-Cardena et al., 1996). Most evidence suggests that tyrosine phosphorylation appears to regulate the interaction of eNOS with caveolin-1 and hence its subcellular localization. Physiologically relevant stimuli such as shear stress appear to stimulate eNOS phosphorylation and increase NO production, in agreement with the known capacity of blood vessels to dilate in response to increased flow. Recently, several studies demonstrated that Ser-1179 of the protein is phosphorylated by protein kinase B/Akt (Dimmeler et al., 1999; Fulton et al., 1999). Again, this phosphorylation was noted to increase NO production. Interestingly, other reports have suggested that HMG-CoA reductase inhibitors, widely used drugs that so effectively lower serum cholesterol, appear to significantly increase the activity of endothelial Akt (Kureishi et al., 2000). These agents, including such widely prescribed agents such as lovastatin and simvastatin, have been shown to have a dramatic effect on cardiovascular mortality. Indeed, their effects on patients’ overall mortality appear to exceed what one would expect from simply lowering cholesterol. As such, a considerable amount of effort has been expended to understand what other potential cardiovascular effects statin therapy might provide. One potential mechanism to alter plaque
progression would be that by raising Akt activity, statins are increasing NO output in the vessel wall and thus potentially providing an additional, cholesterol-independent, benefit to patients. (Fig. 1.3).

Besides the eNOS isoform, two other NOS isoforms have been described and extensively studied. Initially thought to be confined to the nervous system, nNOS (NOS1) was actually the first of the isoforms to be purified and cloned (Bredt et al., 1991). Despite its name, it is clear that nNOS is expressed outside the central and peripheral nervous system and, more specific to our discussion, nNOS is expressed in endothelial and vascular smooth muscle cells (Papapetropoulos et al., 1997; Boulanger et al., 1998) as well as in human atherosclerotic lesions (Wilcox et al., 1997). Nonetheless, the functional significance and role of nNOS in atherosclerosis remain unclear.

The expression of the inducible form of NOS, iNOS (NOS2), is best characterized in inflammatory cells. These cells are very abundant in atherosclerotic lesions. In addition, iNOS upregulation in smooth muscle cells contributes to an overall increase in production of NO in atherosclerosis. A recent study has shown colocalization of this upregulated iNOS with epitopes of oxidized LDL and peroxynitrite-modified proteins (Luoma et al., 1998). It is important to note that the level of NO production from the iNOS isoform is several orders of magnitude higher than from either the eNOS or nNOS isoforms.

The notion that a diffusible gas such as NO could function as a physiological regulator of vascular tone suggests that there are direct and specific protein targets of NO. Evidence suggests that the principal target of endothelium-produced NO is the inactive form of guanylate cyclase located in the underlying vascular smooth muscle cells. The NO-

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**Figure 1.3.** *Cholesterol-dependent and-independent effects of statin therapy.* The widely prescribed class of cholesterol-lowering agents referred to as statins appear to lower death rates from cardiovascular disease to a greater extent than would be predicted by the amount of cholesterol lowering obtained. The search for these cholesterol independent effects include augmenting NO levels by modulating the Akt kinase.
dependent conversion from the inactive to active form of guanylate cyclase in turn catalyzes the production of cGMP from GTP. This causes the relaxation of the smooth muscle cell and subsequent vasodilatation (Fig. 1.4). Although guanylate cyclase represents an important target of NO, many other proteins containing transition metals such as iron, zinc, or copper can also be regulated by NO. The molecular basis for this regulation differs for each target. In the case of guanylate cyclase, NO attacks the bond between His-105 and the ferrous iron associated with the enzyme. This leads to the activation of the enzyme. Other metal-containing proteins that serve as important NO targets include hemoglobin, which appears to be a major intravascular carrier of both molecular oxygen and NO. In addition, a host of transcription factors such as the large family of zinc finger proteins also can be functionally altered by NO exposure. In general, such exposure leads to a decrease in DNA binding, which stands in contrast to the case of guanylate cyclase, where NO exposure activates the enzyme. Finally, other important targets of NO are the enzymes involved in aerobic respiration that contain Fe-S
centers. These enzymes such as aconitase are also subject to inactivation by other ROS and in particular are rapidly inactivated by exposure to superoxide anions.

The mechanisms by which a rise in cGMP and the subsequent activation of the cGMP-activated protein kinase G (PKG) produce a change in vascular tone are the subject of considerable interest and debate (Lincoln et al., 2001). There are at least two major pathways that contribute to the NO-induced vasorelaxation (Fig. 1.5). The first mechanism involves a reduction in intracellular calcium concentrations in vascular smooth muscle. This reduction in calcium is achieved through a number of distinct mechanisms. Recent evidence has demonstrated that the IP3 receptor is a direct target of PKG. Phosphorylation of the smooth muscle IP3 receptor results in a decrease in calcium release from the sarcoplasmic reticulum. In addition, calcium levels are also modulated by the effect of PKG on a number of calcium pumps and voltage-gated channels.

Besides altering calcium levels, the rise in cGMP and the activation of PKG directly alters actin-myosin kinetics. A number of studies have addressed the regulation of myosin light chain (MLC) phosphorylation at Ser-19. Two enzymes with antagonistic functions are primarily responsible for the degree of MLC phosphorylation. These enzymes are myosin light chain kinase (MLCK) and MLC phosphatase. The level of regulatory MLC phosphorylation in turn determines the level of force production and hence the degree of vascular tone. Most available evidence suggests that PKG functions to increase MLC phosphatase activity, producing a decrease in MLC phosphorylation. This decrease in MLC

Figure 1.5. NO-regulated smooth muscle tone through multiple mechanisms. Included among these effects are the activation of protein kinase G (PKG), which in turn alters intracellular calcium levels and myofilament calcium sensitivity.