Nitric Oxide and Mitochondrial Signaling
From Physiology to Pathophysiology
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Abstract—Nitric oxide (NO) has been known for many years to bind to cytochrome C oxidase, the terminal acceptor in the mitochondrial electron transport chain, in competition with oxygen. This interaction may be significant in vivo and explain some of the biological actions of NO. In this article we review the evidence showing that binding of NO to cytochrome C oxidase elicits intracellular signaling events, including the diversion of oxygen to nonrespiratory substrates and the generation of reactive oxygen species. We discuss findings indicating that these NO-elicited events act as triggers by which mitochondria modulate signal transduction cascades involved in the induction of cellular defense mechanisms and adaptive responses. We also discuss instances in which the effects of NO on the electron transport chain might lead to mitochondrial dysfunction and pathology. (Arterioscler Thromb Vasc Biol. 2007;27:2524-2531.)

Key Words: nitric oxide ■ mitochondria ■ reactive oxygen species ■ signal transduction ■ cytoprotection

The roles of mitochondria in the generation of energy,1 the production of reactive oxygen species (ROS),2 the regulation of calcium homeostasis,3 and the orchestration of apoptosis4 are all widely recognized. More recently, evidence has begun to emerge suggesting that these organelles may act also as signaling devices for the regulation of cytoprotective mechanisms and adaptive responses to hypoxia. Earlier observations made by various laboratories indicating that nitric oxide (NO) at physiological concentrations modulates respiration through the reversible inhibition of the mitochondrial enzyme cytochrome C oxidase, in competition with O2,5–7 gave rise to the hypothesis that NO may have an important function in the regulation of such mechanisms.8 Experimental support for this hypothesis comes from a number of studies showing that the interaction of NO with cytochrome C oxidase in different types of cells is associated with the resistance to apoptosis induced by various kinds of stressors, including growth factor deprivation,9 treatment with staurosporine,9,10 O2 limitation,10 or intracellular calcium overload.11 Depending on the system under study, protection was shown to be associated with an increase in mitochondrial membrane potential (Δψm),9 with an increase in glycolytic output linked to upregulation of AMP-activated protein kinase (AMPK)12 or with changes in calcium efflux leading to the induction of the cytoprotective chaperone protein Grp78.11 Further studies also showed that competition between NO and O2 at the level of cytochrome C oxidase is responsible for the inhibition of hypoxia-inducible factor
(HIF) 1-α stabilization observed in the presence of NO under otherwise limiting O₂ concentrations, suggesting that mitochondria under the influence of NO may also be involved in the attenuation of adaptive responses to low O₂. In addition, there is evidence that NO promotes mitochondrial biogenesis by a mechanism that is independent of cytochrome C oxidase but involves activation of the soluble guanylate cyclase.

Excessive production of NO and mitochondrial dysfunction have for many years been independently associated with pathophysiological mechanisms. However, the fact that NO inhibits mitochondrial respiration suggests that there may be instances in which NO production, mitochondrial dysfunction, and pathology could be intimately related. In this review we examine the biochemical actions of NO on mitochondria, their signaling consequences, and their possible relationship to cellular homeostasis and pathophysiology.

**Interaction of Cytochrome C Oxidase With Nitric Oxide**

Cytochrome C oxidase is situated on the inner membrane of the mitochondrion, where it catalyzes the oxidation of cytochrome C and the reduction of O₂ to water in a process linked to the pumping of protons out of the mitochondrial matrix. The enzyme contains 2 heme (a and a₃) and 2 copper centers (Cu₉ and Cu₈), of which the heme iron of cytochrome a₃ together with Cu₈, in their reduced form, form the O₂-binding site. NO closely resembles O₂ and therefore can also bind to this site. In the mid 1990s it was demonstrated that NO inhibits the activity of cytochrome C oxidase. This inhibitory effect was shown to be reversible, in competition with O₂, and to occur at concentrations of NO likely to be present physiologically. Thus for example, at 30 μmol/L O₂ (approximately the tissue concentration of O₂) the IC₅₀ of NO for cytochrome C oxidase is 60 nmol/L, whereas at 10 μmol/L (a possible intracellular concentration of O₂) the IC₅₀ of NO for the enzyme would be predicted to be approximately 20 nmol/L. In addition, it has recently been reported that the Kᵣ of NO for the O₂-binding site of cytochrome C oxidase is ≈0.2 nmol/L, confirming that concentrations of NO that have been detected in tissues (10 to 450 nmol/L) would be sufficient to compete with intracellular O₂.

The potential biological relevance of the NO-cytochrome C oxidase interaction has been further highlighted by a number of studies demonstrating inhibition of respiration by endogenously-generated NO, or its enhancement by inhibitors of NOS in a number of cells, isolated tissues, and whole animals. In studies with vascular endothelial cells in culture it was found that endogenous concentrations of NO modulate cell respiration in an oxygen-dependent manner. Furthermore, treatment with the neuropeptide bradykinin, which activates the endothelial isoform of NO synthase (eNOS), generated concentrations of NO that inhibited respiration further. Conversely, treatment with an inhibitor of NOS resulted in an immediate increase in O₂ consumption, suggesting that endogenous NO interacts with cytochrome C oxidase and modulates O₂ consumption under basal and stimulated conditions.

Recently we found that, when cells respire to anoxia in a closed chamber, endogenous NO induces a reduction of cytochromes cc₁ and aa₃ of the electron transport chain before inhibition of respiration is observed. Consistent with studies with isolated cytochrome C oxidase, further work using intact cells suggests that NO interacts with the enzyme in two ways. In the first case, which occurs at high [O₂] and low electron turnover in the enzyme, NO interacts primarily with the prevailing oxidized species of the catalytic cytochrome C oxidase cycle, resulting in an increase in the reduced fraction of cytochromes cc and consequently a rise in the reductive pressure on the NO-free fraction of the enzyme. This situation, in turn, causes an increase in the electron turnover of the uninhibited fraction of the enzyme, thus allowing for steady state respiration to be maintained. The second case takes place at low [O₂], and possibly also at high [O₂] if NO levels rise above the physiological nmol/L range. Under these conditions, which favor a high electron turnover, the high affinity interaction of NO with the reduced species of the catalytic cycle will result in inhibition of respiration.

**Biochemical Events Initiated by the Interaction Between Nitric Oxide and Cytochrome C Oxidase**

**Generation of Reactive Oxygen Species by Mitochondria**

Experimental evidence accumulated between the late 1960s and late 1970s suggests that a small percentage of the O₂ used by mitochondria is not completely reduced to water but is converted to O₂•⁻ because of the escape of electrons at complexes I and III of the electron transport chain. Theoretical considerations and experimental evidence indicate that the redox state of the mitochondrial respiratory chain may be a major determinant in the control of this process (reviewed by Turrens). Studies using carbon monoxide have also suggested that the reduction of the electron transport chain as a consequence of cytochrome C oxidase inhibition may enhance O₂•⁻ formation. Studies in isolated mitochondria have indicated that treatment with NO generates O₂•⁻ in a similar manner. We have investigated this phenomenon in more detail using a recently-developed polarographic-and-spectroscopic-coupled system based on visible light spectroscopy (VLS). This system enables us to monitor in real-time the effects of endogenous NO on changes in the redox state of the electron transport chain as they occur during respiration in a closed system. Working with the monocytic cell line RAW 264.7 and with human umbilical vein endothelial cells (HUVECs) we found that, when the O₂ concentration reached ≈50 μmol/L (a physiologically relevant level), there was a reduction of cytochromes aa and cc of the electron transport chain although the rate of O₂ consumption was still maximal. This phenomenon, which had previously been reported by other laboratories both in intact cells and in vivo, appears to be modulated by endogenous basal levels of NO; indeed the early reduction of the cytochromes was only in evidence at much lower [O₂] when the NOS inhibitor N⁶-monomethyl-L-arginine (L-NMMA) was present. This observation led us to speculate, and then to confirm experimentally, that if NO levels are raised, the early reduction of the cytochromes will be observed at higher [O₂]. Thus, these
experiments suggest that NO acts as a rheostat that sets the concentration of O$_2$ at which an early reduction of the electron transport chain will occur without inhibition of respiration. In parallel experiments we also found that when RAW 246.7 cells and HUVECs are incubated at 3% O$_2$ (giving ~30 μmol/L O$_2$ in the culture medium), the early reduction of the electron transport chain correlates with an NO-dependent increase in O$_2$- release observed at decreasing [O$_2$].

Conversely, there are many reports indicating that NO causes mitochondrial membrane depolarization in association with the induction of apoptosis. Although some of these seemingly contradictory observations may be attributed to the methodology used to detect changes in ∆ψm, there may be cases in which these opposing actions of NO may result from differences in the metabolic or redox environment of the target cell.

**Activation of AMP-Kinase**

Insufficient energy output results in bioenergetic crisis. This phenomenon may stem from a variety of biological situations, including increased energy demand, restriction of nutrient or oxygen supply (ischemia and hypoxia), and mitochondrial dysfunction. Bioenergetic crisis causes an increase in intracellular AMP levels, and this in turn leads to the activation of the AMP-activated protein kinase (AMPK), an enzyme which plays a central role in the control of intracellular energy metabolism.45 AMP binding to the enzyme promotes its phosphorylation by the tumor suppressor LKB1, resulting in full activation.46 Once activated, the enzyme turns off biosynthetic pathways and at the same time turns on catabolic pathways, thus conserving ATP levels.

Because of the important role played by AMPK in the regulation of energy metabolism, we recently began to study the role of mitochondria-mediated NO signaling in the functioning of this enzyme. In our initial studies we observed that AMPK was activated when HUVECs were cultured at 3% O$_2$.47 We then found that this effect was dependent on the presence of endogenous NO because it could be abolished by L-NMMA47 or when endothelial NO synthase (eNOS) expression was partially reduced by RNA interference (Colombo and Moncada, 2006). Interestingly, the activation of AMPK observed at 3% O$_2$ was not attributable to an energetic crisis (ie, there was no increase in AMP levels); instead, it appeared to be mediated by an increase in mitochondria-derived ROS, because the effect could be abolished by depletion of mitochondrial DNA or by coincubation with antioxidants.47 These findings are consistent with observations in other systems indicating that, in addition to AMP, the enzyme can be activated by ROS generated from a variety of sources,48–50 and with the fact that treatment of endothelial cells with NO donors or with fluid shear stress, leads to activation of AMPK.51 In addition, we have recently found that, in contrast to a number of other tissues which express both isoforms (α1 and α2) of the AMPK catalytic subunit,52 HUVECs only express the α1 isoform (Colombo and Moncada, 2006). Although there is clear evidence that the activation of AMPK complexes containing the α2 subunit is dependent on a bioenergetic crisis,53 complexes containing the α1 subunit seem to be far less responsive to changes in AMP. Our experiments are in agreement with this observation, because only very substantial increases in AMP activated AMPK in HUVECs.47 Whether there are differences in downstream targets activated by each of the catalytic subunits is unknown at present. Similarly, the precise biochemical mechanism responsible for the activation of AMPK by ROS remains to be elucidated.

**Modulation of Mitochondrial Membrane Potential**

Studies in intact lymphoid cells and astrocytes showed that inhibition of respiration by NO results in a temporary small decrease in ∆ψm. This phenomenon depends on the capacity of some cell types to maintain ATP levels by glycolysis when respiration is compromised. Generation of a ∆ψm under these conditions requires entry of the glycolytically-generated ATP to the mitochondrial matrix via the adenine nucleotide translocator and its subsequent hydrolysis by the F$_0$F$_1$ ATPase which, now acting in reverse, extrudes protons from the mitochondrial matrix. An increase in ∆ψm has previously been detected in association with the initiation of apoptosis.41 The possibility that NO may also be involved in this phenomenon is underscored by findings showing that several proapoptotic factors stimulate NO production.42,43 Furthermore, the possibility that a high ∆ψm promotes the formation of O$_2$- by complex III,44 suggests that this force may also contribute to the NO-stimulated increase in O$_2$- release observed at decreasing [O$_2$].
Modulation of Glycolytic Output

AMPK activation is known to enhance glucose uptake and glycolysis under physiological (e.g., endurance exercise training) and pathological contexts (e.g., cardiac ischemia) involving a decrease in intracellular O2 levels. Regulation of these processes by AMPK occurs at multiple levels, including transcription, posttranslational phosphorylation, and intracellular localization. Thus, enhancement of glucose uptake appears to be mediated by increasing gene transcription of glucose transporters and by promoting their translocation to the cell surface. Upregulation of glycolysis results primarily from the phosphorylation and activation of 6-phosphofructo-2-kinase (PFK-2), the enzyme responsible for the synthesis of fructose 2,6-bisphosphate, a potent stimulator of glycolysis. In addition, AMPK activation is known to upregulate the expression of hexokinase II.

Recent evidence indicates that, in astrocytes, inhibition of mitochondrial respiration by NO leads to the upregulation of glycolysis, affording cytoprotection against energy failure. Both these effects are mediated via stimulation of AMPK and without the involvement of guanylate cyclase (reviewed by Moncada and Bolan˜os59). Indeed, similar to its effects in muscle cells, active AMPK stimulates PFK-2 activity in astrocytes leading to the upregulation of PFK-1. In addition, it causes an enhancement of glucose uptake via upregulation of GLUT3 expression.60 These findings suggest that NO-induced mitochondrial signaling might also play a role in the AMPK-mediated enhancement of glycolytic output in other cell types, in which pathophysiological situations lead to the development of moderate cellular hypoxia. Furthermore, the finding that NOS is also a downstream target of AMPK in heart cells61 raises the possibility that NO could reinforce the activity of this enzyme in these cells via an autostimulatory loop.

Diversion of Oxygen to Nonrespiratory Substrates

We have previously postulated that the interaction of NO with cytochrome C oxidase may result in “metabolic hypoxia”, a situation in which, although there might be enough O2 available to sustain respiration, the presence of NO prevents the cell from using it for this purpose.6 A consequence of this phenomenon would be an increased availability of O2 in subcellular compartments outside the mitochondrion. For example, the firefly depends on this action of NO to produce light.62 Using the human embryonic kidney cell line HEK293 as a model system, we found that NO also redistributes O2 away from the mitochondrion in mammalian cells, toward nonrespiratory O2-dependent targets, in this case the prolyl hydroxylases involved in the degradation of HIF-1α.13 Furthermore, in HEK293 cells, we subsequently found that NO prevents the stabilization of HIF-1α under moderate hypoxia (1.5% to 3% O2) in HUVECs.63 Consistent with our findings, inhibition of respiration by oligomycin64 and inhibitors of the electron transport chain65 was recently shown to diminish HIF-1α accumulation in tumor cells.

Modulation of Mitochondrial Calcium Release

Experiments using cell lines have shown that inhibition of mitochondrial respiration by endogenously-generated NO may cause changes in mitochondrial calcium fluxes which lead to the activation of ATF6, a transcription factor associated with the endoplasmic reticulum stress response.6 Nitric oxide–induced release of mitochondrial calcium has also been observed in neurons, an effect which in this case appears to be preceded by the loss of Δψm (reviewed by Moncada and Bolan˜os59). The precise biological significance of the NO-induced mitochondrial calcium fluxes is unclear at present. In this regard, recent evidence suggesting that AMPK may also be activated by the Ca2+-calmodulin-dependent kinase kinase in a manner that is dependent on bioenergetic changes,65,66 points to an additional mechanism in which mitochondria-mediated NO signaling may regulate the AMPK.

Modulation of Transcription Factor Activity

Diversion of O2 to the cytoplasm leading to the downregulation of HIF-1α, and the release of mitochondrial calcium resulting in the activation of the transcription factor ATF6, are two clear examples by which NO may affect gene expression through its effects on mitochondria. Circumstantial evidence suggests that NO-stimulated mitochondrial signaling may regulate additional transcription factors, particularly those that respond to redox signals. Indeed, when RAW 246.7 cells are incubated at 3% O2, the early reduction of the electron transport chain not only correlates with an NO-dependent increase in O2 levels but also with the migration to the nucleus of the transcription factor NF-κB.23 This raises the possibility that other redox-sensitive transcription factors may be affected in a similar fashion. Among these, regulation of NF-E2 related factor (Nrf-2) (reviewed by Nguyen et al67) may be of relevance because of its involvement in the constitutive expression of cytoprotective genes under physiological conditions.68,69 Furthermore, in endothelial cells, NO has been shown to stimulate Nrf-2 nuclear translocation via a mechanism that is sensitive to antioxidants and to inhibitors of mitogen-activated protein kinase (MAPK) pathways.70 In addition, it has been demonstrated that laminar flow, which increases eNOS activation71 and expression,72 also induces the expression of Nrf-2 target genes.73 However, the involvement of mitochondrial-derived ROS in these responses remains to be examined.

Recent evidence suggests that in endothelial cells AMPK activation by laminar flow results in the phosphorylation and concomitant degradation of the forkhead transcription factor FoxO1.15 Again, although NO was instrumental in the induction of these effects, a mitochondrial link has yet to be established. FoxO1 belongs to a subfamily of transcription factors which are known to induce a wide array of target genes, and which have been implicated in diverse cellular and physiological functions, including differentiation, cell cycle arrest, apoptosis, tumor suppression, glucose metabolism, adaptation to oxidative stress, and the regulation of lifespan.74 Regulation of FoxO-mediated gene transcription is orchestrated by multiple phosphorylation signals, including negative regulation by the PI3K/AKT pathway and positive regulation by the stress-activated protein kinase JNK, as well as by interaction with different cotranscriptional regulators. In addition, deacetylation by Sir-2 family deacetylases appears to play an important role in tipping the balance away from the induction of cell death to the induction of stress resistance. Whether NO-elicited mitochondrial signaling plays a role in the modulation of these responses has yet to be explored.
Functional Consequences of Nitric Oxide–Stimulated Mitochondrial Signaling

Induction of Cytoprotection

The consequences of the biochemical events elicited by the interaction between NO and cytochrome C oxidase are now beginning to be clarified. Currently available evidence suggests that this interaction and the consequent shift in the redox balance of the mitochondrial electron transport chain to a more reduced state favors the generation of \( \text{O}_2^- \), which could be subsequently converted to H\(_2\)O\(_2\) by SOD. H\(_2\)O\(_2\) in turn may act as a second messenger, which, directly or indirectly (via protein phosphorylation) and simultaneously, stimulates various signaling pathways, including those involving AMPK activation and nuclear migration of redox-sensitive transcription factors. Transient calcium fluxes from the mitochondrion may also be involved in these processes. The activation of AMPK may in turn lead to an increase in the activity of 6-phosphofructo-1-kinase which maintains a high glycolytic output. At the same time, active AMPK, JNK, nuclear NF-\(\kappa\)B, and other redox-sensitive transcription factors may participate in concert in the upregulation of cytoprotective genes, including those encoding glucose transporters, antiapoptotic factors and enzymes involved in glycolysis, antioxidant defenses, and repair mechanisms. Other redox-sensitive signaling proteins which localize to mitochondria, such as the tyrosine kinase c-src\(^7\) and the protein tyrosine phosphatase Shp-2\(^7\) could also be involved, but their role in the mitochondrial responses to NO has yet to be investigated. In addition, when \( \text{O}_2 \) tension falls to the lower end of the physiological range, NO may divert \( \text{O}_2 \) to the cytosol, favoring the degradation of HIF-1\(\alpha\).

Signaling from the mitochondrion to the nucleus is known to occur as a consequence of mitochondrial dysfunction in yeast and mammalian cells.\(^7\) Though reminiscent of some events elicited by the action of NO on cytochrome C oxidase, such signaling represents a stress-response mechanism. In contrast, the NO-elicited mitochondrial signaling events described above are likely to be part of a constitutive cytoprotective house-keeping mechanism, arising from the involvement of the mitochondrial in physiological homeostasis. Despite these differences, some downstream effectors, such as NF-\(\kappa\)B, may be common to both mechanisms.\(^23,79\) In this respect, NF-\(\kappa\)B has not only been associated with pathological consequences,\(^79,80\) but also with cytoprotective responses,\(^81–84\); these opposing roles depend in part on the signaling context and probably on the magnitude of the activation.

A schematic representation of the signaling pathways that may be activated as a consequence of the interaction between NO and cytochrome C oxidase to confer cytoprotection is shown in the Figure.

Cytoprotection in Endothelial Cells

The precise nature and outcome of the signaling events described above may depend on the individual cellular and physiological context. In the particular case of endothelial cells, we propose that NO-elicited mitochondrial signals may be involved in maintaining a “constitutive defensive phenotype” with 2 component parts. One of these would depend on the NO-induced generation of mitochondrial \( \text{O}_2^- \) which would act as a second messenger to maintain the constitutive level of antioxidant and other cytoprotective enzymes relatively high. The other would depend on the diversion of \( \text{O}_2 \) to the cytosol and will ensure that endothelial cells are prevented from entering into an angiogenic phenotype at the low end of the physiological \( \text{O}_2 \) concentration range. We also suggest that the constitutive defensive phenotype may be compromised in situations of reduced NO availability, rendering endothelial cells susceptible to apoptosis, accelerated senescence, or pathological angiogenesis, depending on the nature of the external challenge to which the cells are subjected.
Other investigators have suggested that NO protects endothelial cells via mechanisms involving S-nitrosation of susceptible proteins, such as caspase-3 and thioredoxin. However, although there is some support for this hypothesis, its relevance to the role of NO in physiological homeostasis is far from clear. There are several reasons for this, including the lack of a clear in vivo correlate of a significant physiological function being regulated by this process and the fact that NO by itself is a very poor nitrosating species.

Endothelial cells are dependent primarily on glycolysis for their energetic demands. Thus, the primary role of mitochondria in these cells may not be related to the supply of energy but rather to the adaptation to external stresses.

**Pathophysiological Consequences**

Reaction between NO and O$_2^*$ results in the formation of peroxynitrite (ONOO\textsuperscript{-}). This process occurs at near-diffusion-limited rates (6.7×10$^9$ M$^{-1}$s$^{-1}$), which are even faster than the dismutation of O$_2^*$ by SOD. Direct measurement of ONOO\textsuperscript{-} generation in biological systems is not possible; however, protein tyrosine nitration, a footprint of the formation of ONOO\textsuperscript{-}, has been found in a number of human pathologies (reviewed by Greenacre and Ichiropoulou). The source of O$_2^*$ involved in the formation of ONOO\textsuperscript{-} has been widely debated. Under physiological conditions, the contribution of mitochondrial O$_2^*$ to this process is improbable because of the high levels of SOD1 and SOD2 present in the intermembrane mitochondrial space and the mitochondrial matrix, respectively. However, reaction between NO and O$_2^*$ generated by the electron transport chain is likely to occur when the systems that effectively dismutate O$_2^*$ are impaired or saturated. The formation of ONOO\textsuperscript{-} can take place only in the vicinity of the enzymes generating O$_2^*$ owing to the short half-life of the latter and the high hydrophobicity of NO. Tyrosine nitration of mitochondrial proteins has been detected in vivo in several situations, including aged rats, experimental immune encephalomyelitis, and traumatic brain injury. Nevertheless, in most instances the biochemical events leading to protein nitration in pathological circumstances have not been completely elucidated.

Long-term exposure of cells to NO leads to a gradual and persistent inhibition of complex I that occurs concomitantly with a reduction of glutathione. This inhibition could be the result of S-nitrosation of critical thiols in complex I, because the effect was reversed by exposure to high intensity light or by replenishing intracellular glutathione. This possibility has now been confirmed by a number of research groups. Mitochondria have been shown to be crucial for the formation of S-nitrosoproteins, possibly because of their capacity to generate O$_2^*$ and, as a consequence, ONOO\textsuperscript{-}. Furthermore, it has recently been shown that S-nitrosation of complex I causes an increase in mitochondrial H$_2$O$_2$ production. It is not clear, however, whether these processes are linked to physiological signaling responses or represent an early sign of oxidative stress and pathology. Thus, for example, it has been claimed that S-nitrosation of complex I is protective in rat hearts subjected to ischemia-reperfusion. Conversely, inactivation of complex I by S-nitrosation has been implicated in Parkinson disease. Whether sequential inhibition of cytochrome C oxidase and complex I occur in conditions such as inflammation and degenerative disease is not known at present.

**Concluding Remarks**

The concept that NO is involved in cytoprotection is not new. Whether this is controlled by the interaction between NO and cytochrome C oxidase is an interesting possibility that requires further examination. Because the majority of experimental work investigating the mechanisms of action of NO has been carried out with cells cultured at ambient [O$_2$], the signaling consequences of the interaction between the enzyme and physiologically meaningful levels of NO have so far remained largely undetected. Future studies carried out in cells kept at 3% O$_2$, a concentration which more closely reflects the situation in vivo, will help to establish the biological significance of this interaction. Knowledge derived from this type of research may help in the future design of new strategies for therapeutic intervention in vascular biology and beyond.

**Acknowledgments**

The authors thank Annie Higgs for help in the preparation of this manuscript.

**Disclosures**

None.

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Arterioscler Thromb Vasc Biol. 2007;27:2524-2531; originally published online September 20, 2007;
doi: 10.1161/ATVBAHA.107.151167
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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