# 4-Hydroxynonenal Prevents NO Production in Vascular Smooth Muscle Cells by Inhibiting Nuclear Factor-κB–Dependent Transcriptional Activation of Inducible NO Synthase

Yoshiyuki Hattori, Sachiko Hattori, Kikuo Kasai

***Abstract***—The role of lipid peroxidation products in atherogenesis was studied. We investigated whether 4-hydroxy-2-nonenal (HNE) modulates activation of the nuclear factor (NF)-κB system or alters expression of the NF-κB target gene product, inducible NO synthase (iNOS), in vascular smooth muscle cells (VSMCs) stimulated by lipopolysaccharide (LPS) in combination with interferon (IFN)-γ (LPS/IFN). NO production induced by LPS/IFN was dose-dependently inhibited by HNE. NF-κB activation by LPS/IFN was inhibited by HNE in a dose-dependent manner. HNE significantly decreased LPS/IFN-stimulated proteolysis of IκB-α. iNOS promoter activity stimulated by LPS/IFN was also decreased by HNE dose-dependently. The treatment of VSMCs with LPS/IFN strongly stimulated iNOS mRNA and protein expression. The LPS/IFN-induced increases in iNOS mRNA and protein levels were dose-dependently decreased by HNE. Our data suggest that treatment with HNE blocks signaling events required for IκB-α degradation, thereby preventing NF-κB activation. Inhibition of NF-κB–regulated gene expression, especially modulation of NO production, may contribute to atherogenesis. (***Arterioscler Thromb Vasc Biol*. 2001;21:1179-1183.)**

**Key Words:** 4-hydroxy-2-nonenal ■ nuclear factor-κB ■ nitric oxide ■ vascular smooth muscle cells

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Atherosclerotic lesion formation is a complex process, which is in part mediated by inflammatory and oxidative mechanisms, including lipid peroxidation. Oxidized LDL has been shown to be present in atherosclerotic lesions.[1,2] Oxidized LDL is a heterogeneous mixture of various components, including a variety of reactive aldehyde products that are formed during the oxidative degradation of polyunsaturated fatty acids.[3] There is increasing evidence that aldehydes generated endogenously during the process of lipid peroxidation are causally involved in most of the pathophysiological effects associated with inflammatory and oxidative stress in cells and tissues.[4] Lipid peroxidation–derived aldehydes appear not only to be end products and remnants of lipid peroxidation processes but also may act as mediators for the primary free radicals that initiate lipid peroxidation. Among the lipid peroxidation–derived aldehydes, 4-hydroxy-2-nonenal (HNE) is believed to be largely responsible for the cytopathological effects observed during inflammatory and oxidative processes.[4]

Nuclear factor (NF)-κB, a pleiotropic transcription factor, has been suggested to play an important role in gene regulation during the inflammatory and immune reactions that promote atherosclerotic lesions.[5–7] NF-κB regulates the inducible expression of a variety of genes involved in inflammatory and immune responses, including inducible NO synthase (iNOS).[8–11] NO appears to play a key role in mediating vascular remodeling.[12–14] HNE-modified lysine residues have been identified in atherosclerotic lesions,[15] and a variety of HNE effects in different cell types have been described.[16–20] HNE may modulate arterial wall NO production in atherosclerotic lesions.

In the present study, we sought to define the effects of HNE on iNOS expression in vascular smooth muscle cells (VSMCs). We investigated whether HNE is able to modulate activation of the NF-κB system or alter the expression of the NF-κB target gene product iNOS. We examined to what extent this aldehyde affects κB-dependent and iNOS promoter–dependent transcriptional activity, the expression of iNOS mRNA and protein, and NO production.

## Methods

### Cell Culture and RNA Extraction

Rat VSMCs were isolated by elastase and collagenase digestion of thoracic aortas from male Wistar rats, as previously described.[21] Cells in passages 10 to 15 were used for experiments. Total RNA was extracted from confluent human VSMCs by using a modified guanidinium isothiocyanate method.[22]

### Nitrite Assay

Nitrite accumulation, an indicator of NO synthesis, was measured in the cell culture medium of confluent VSMCs.[23] Nitrite was quanti-

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fied colorimetrically after adding 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid) to 100-μL samples. Absorbance at 550 nm was determined by using a microplate reader (Molecular Devices). Nitrite concentrations were calculated by comparison with the absorbance of standard solutions of sodium nitrite prepared in cell culture medium.

## Cell Respiration

Cell respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan.[24] To examine the cytotoxic effect of HNE, the cells were incubated (37°C) with MTT (0.4 mg/mL) for a further 60 minutes after a 24-hour incubation for nitrite assay. Culture medium was removed by aspiration, and the cells were solubilized in dimethyl sulfoxide. The extent of reduction of MTT to formazan within cells was quantified by the measurement of optical density at 550 nm. Formazan production is compared with the values obtained from the control cells (no HNE).

## iNOS mRNA Analysis

An iNOS cDNA was kindly provided by Dr Y. Nunokawa[24a] (Suntory Co, Osaka, Japan) and labeled with [$\alpha$-$^{32}$P]dCTP by random priming for use as a Northern blot probe to assess iNOS mRNA expression. Northern blot procedures were performed as previously described.[25] After probing for iNOS expression, filters were stripped and reprobed for the presence of GAPDH mRNA. The blots were exposed to an imaging plate (Fuji Photo Film Co) at room temperature for 6 hours and analyzed by using a FUJIX bioimaging analyzer (BAS2000II, Fuji Photo Film Co).

## NF-κB Activation

To study NF-κB activation, the cells were stably transfected with a *cis*-reporter plasmid containing a luciferase reporter gene linked to 5 repeats of NF-κB binding sites (pNFκB-Luc, Stratagene).[25] For this, the pNFκB-Luc plasmid was transfected together with a pSV2neo helper plasmid (Clontech) into rat VSMCs by using the FuGEN 6 transfection reagent (Boehringer-Mannheim). The cells were cultured in the presence of G418 (Clontech) at a concentration of 500 μg/mL with medium replacement at 2- to 3-day intervals. Approximately 3 weeks later, G418-resistant clones were isolated by using a cloning cylinder and analyzed individually for expression of luciferase activity. Several clones were selected for analysis of NF-κB activation. Luciferase activity was measured by using a luciferase assay kit (Stratagene).

## iNOS Promoter Activity Assay

To study iNOS promoter function, VSMCs were stably transfected with a construct containing a 1.7-kb fragment of the iNOS promoter, which was cloned in front of a reporter gene encoding secreted alkaline phosphatase (SEAP), as previously described.[26,27] SEAP activity, released into the cell culture medium, was measured by a sensitive chemiluminescent assay (Phospha-Light, TROPIX).

## Western Blot of iNOS and IκB-α

After treatment, VSMC monolayers were lysed by using an electrophoresis sample buffer. The protein concentration of the samples was measured by use of a Bio-Rad detergent-compatible protein assay. Subsequently, β-mercaptoethanol was added to the samples at a final concentration of 1%, and samples were denatured by boiling for 5 minutes. Samples containing 10 μg of protein were electrophoresed on 7% SDS-polyacrylamide gels and transferred to PVD membranes (Bio-Rad), which were incubated with a rabbit polyclonal antibody against human IκB-α (1:2000, Santa Cruz Biotechnology) or a mouse monoclonal IgG1 antibody against mouse iNOS (1:2000, Transduction Laboratories). IκB-α antibody or iNOS antibody binding was detected by using donkey anti-rabbit or sheep anti-mouse IgG horseradish peroxidase (1:20 000) and the ECL Plus system (Amersham).

## Statistical Analysis

Data are presented as mean±SEM of measurements in 2 or 3 different VSMC cell cultures. Multiple comparisons were evaluated by ANOVA followed by the Fisher protected least significant difference test. The Student unpaired *t* test was used for comparisons between 2 experiments. A value of $P<0.05$ was considered statistically significant.

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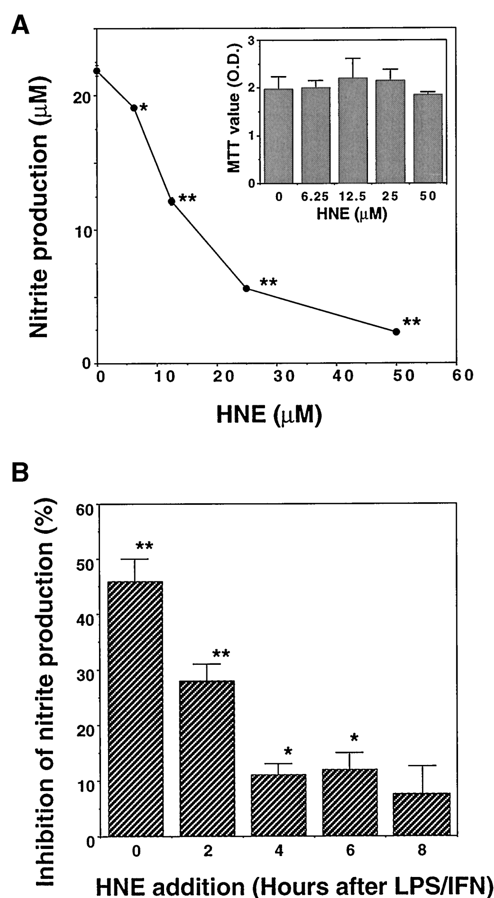


**Figure 1.** A, Effect of HNE on nitrite production in LPS/IFN-stimulated rat VSMCs. Cells were treated with a combination of LPS/IFN in the presence of various concentrations of HNE for 24 hours, after which nitrite accumulation in the culture medium was measured. Data are mean±SE (n=9). *$P<0.05$ and **$P<0.01$ vs LPS/IFN in the absence of HNE. Nitrite was undetected in the medium from unstimulated cells. Inset, Effect of HNE on cellular respiration. MTT values were measured 24 hours after cells were stimulated with LPS/IFN. B, Effect of adding HNE after stimulation of rat VSMCs with LPS/IFN. HNE (25 μmol/L) was added at the indicated times after stimulation. Nitrite accumulation was measured 24 hours after LPS/IFN. Data are mean±SE (n=6). *$P<0.05$ and **$P<0.01$ compared with control (no HNE).

# Results

Our initial studies characterized NO production in VSMCs by determining the cumulative production of nitrite in culture supernatants at various intervals after treatment with lipopolysaccharide (LPS) in combination with interferon (IFN)-γ (LPS/IFN). A lag phase of 6 to 8 hours preceded the induction of nitrite synthesis, followed by a progressive increase in nitrite synthesis for at least 24 hours.

Studies with different concentrations of HNE (5 to 100 μmol/L) showed a concentration-dependent inhibition of nitrite production when HNE was added to VSMCs before LPS/IFN (Figure 1a). Although 50 μmol/L HNE produced an ≈70% to 75% decline in nitrite production after LPS/IFN stimulation, 5 μmol/L HNE had little effect. Our cytotoxicity

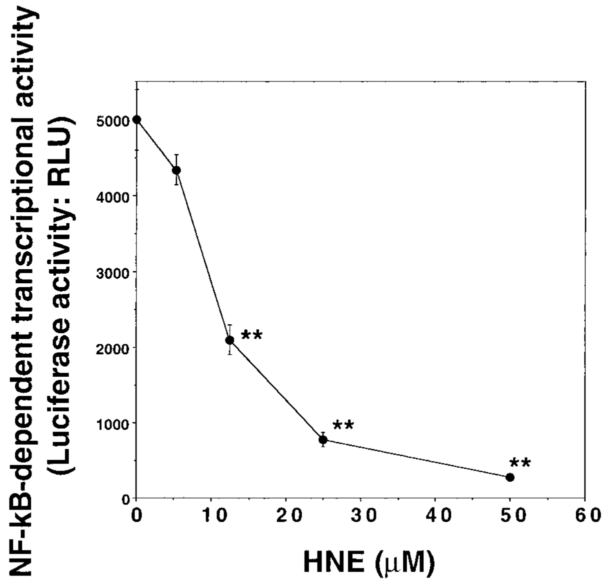


**Figure 2.** Effect of HNE on NF-κB–dependent transcriptional activity/luciferase reporter expression in stably transfected rat VSMCs. Cells were treated with LPS/IFN in the presence of various concentrations of HNE for 3 hours. After treatment, luciferase activity in the cells was measured. RLU indicates relative light units. Values are mean±SE (n=6). $^{**}P<0.01$ vs LPS/IFN in the absence of each agent.

studies showed that even at the highest concentration of HNE, only a marginal reduction in cell viability was achieved (ie, there was little effect on MTT values compared with the effect in the absence of HNE; inset to Figure 1a).

Time-course studies using a final concentration of 25 μmol/L HNE were conducted to determine whether HNE had a direct affect on VSMC NO synthase (NOS) activity, ie, altered NOS expression by VSMCs (Figure 1b). When HNE was added at increasing intervals after the stimulation of VSMCs with LPS/IFN, the inhibition of nitrite production decreased as the interval lengthened.

Next, experiments were performed to examine whether HNE affects activation of the NF-κB system. NF-κB activation was detected by measuring NF-κB–dependent transcription in rat VSMCs stably transfected with a luciferase reporter construct. Cells were treated with different concentrations of HNE and then stimulated with LPS/IFN for 3 hours. In the absence of HNE, we observed the expected activation of NF-κB by LPS/IFN. This increase was slightly affected by HNE at 6.25 μmol/L, significantly decreased by 12.5 and 25 μmol/L, and completely abolished by 50 μmol/L. No effect of HNE alone on NF-κB activity was observed (Figure 2). We then examined whether HNE could affect the activation-induced proteolysis of the IκB inhibitor protein that traps the NF-κB dimer in the cytosol. Incubation of VSMCs with LPS/IFN over 1 hour led to a significant proteolysis of IκB-α. HNE, when added before LPS/IFN, significantly decreased LPS/IFN-stimulated proteolysis of IκB-α (Figure 3A). Pretreatment with the proteosome inhibitor MG115 also inhibited IκB-α degradation (Figure 3B).

We also tested whether HNE modulates activation of the iNOS promoter. iNOS promoter function was determined by measuring iNOS promoter–dependent transcription in rat VSMCs stably transfected with a SEAP reporter construct

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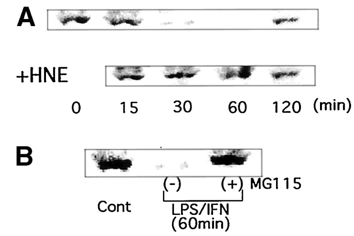


**Figure 3.** Effect of HNE and a proteosome inhibitor on degradation of IκB-α by LPS/IFN in rat VSMCs. A, Cells were treated with HNE (50 μmol/L) and stimulated with LPS/IFN for the indicated time. B, Cells were pretreated with MG115 (10 μmol/L) for 60 minutes and stimulated with LPS/IFN for 60 minutes. The extracted cell lysates were subjected to Western blot analysis with use of an anti–IκB-α antibody. Cont indicates control.

containing the iNOS promoter. iNOS promoter activity increased in the cells treated with LPS/IFN by ≈5-fold relative to unstimulated levels. This increase was dose-dependently decreased by HNE (Figure 4).

Next, we investigated whether the inhibition of transcriptional activity by HNE has a corresponding impact on gene and protein expression. For this purpose, we evaluated iNOS mRNA levels and iNOS protein levels by Northern and Western blot analyses and NO production by monitoring the accumulation of nitrite in the cell supernatant. Although iNOS mRNA levels approached the detection limit by Northern blot analysis in unstimulated VSMCs, treatment with LPS/IFN provided a strong stimulus for iNOS mRNA expression. The LPS/IFN-induced increase in iNOS mRNA level was dose-dependently decreased by HNE (Figure 5). As shown in Figure 6, iNOS protein levels were very low in unstimulated cells. Treatment with LPS/IFN caused a substantial induction of iNOS protein expression in the cells. This induction of iNOS protein was significantly decreased by HNE at 12.5 μmol/L and abolished at 50 μmol/L.

## Discussion

The present report demonstrates that HNE, one of the most prominent aldehyde substances generated during extensive lipid peroxidation of oxidized LDL, inhibits the activation of

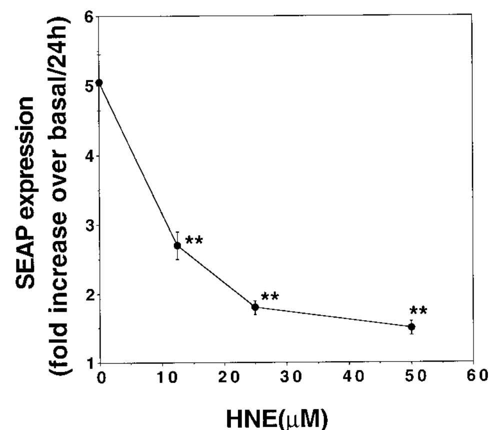


**Figure 4.** Effect of HNE on iNOS promoter/SEAP reporter expression in stably transfected rat VSMCs. Cells were treated with LPS/IFN in the presence of various concentrations of HNE for 24 hours. After this time, SEAP activity was quantified in the cell culture medium. Values are mean±SE (n=6). $^{**}P<0.01$ vs LPS/IFN alone.

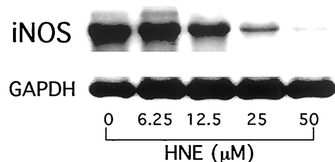


**Figure 5.** Effect of HNE on iNOS mRNA expression in rat aortic smooth muscle cells. Cells were treated with LPS/IFN in the presence of various concentrations of HNE. Total RNA was isolated and analyzed by Northern blot hybridization with a rat iNOS-specific probe.

NF-κB and κB-dependent transcription in VSMCs. Furthermore, iNOS promoter–dependent transcription and the induction of iNOS mRNA and protein were dose-dependently reduced in the presence of HNE, which implies that the effect of HNE on the NF-κB system is associated with functional consequences. Indeed, HNE inhibited the induction of NO production in VSMCs stimulated with LPS/IFN.

Our data show that HNE prevented the degradation of the cytosolic NF-κB inhibitor protein IκB-α after LPS/IFN stimulation. Our cytotoxicity studies show that even at the highest concentration of HNE, only a marginal reduction in cell viability was achieved. IκB proteolysis is mediated by the proteosome,[28] and our data show that a proteosome inhibitor blocked degradation of IκB. Thus, the inhibitory effect of HNE on NF-κB is not due to general cytotoxicity, but HNE appears to selectively block signaling events that are required for IκB degradation, thereby preventing NF-κB activation. It has been reported that in cells of the monocytic lineage, HNE specifically prevents LPS-induced phosphorylation of IκB-α, whereas the profound effect of HNE on NF-κB is not due to interference with the LPS-binding properties of the cells.[20] The authors suggest that HNE appears to inhibit IκB phosphorylation at a signaling stage located downstream from the LPS receptor level. This is also likely to be one of the mechanisms involved in HNE inhibition of the NF-κB system in VSMCs, and the detailed mechanism by which this occurs, including HNE-induced IκB degradation in stimulated VSMCs, is currently under investigation.

Atherosclerosis is associated with reduced endothelium-dependent vasodilation, which has been ascribed to reduced NO formation.[29,30] Constitutively expressed endothelial NOS is responsible for the regulated production of NO in arteries, whereas iNOS is expressed under inflammatory conditions.[12–14] The loss of endothelial NOS may be compensated for by increased iNOS expression. As shown previously, HNE is present in atherosclerotic lesions.[2,15] Because HNE can cause oxidative stress and inhibit iNOS, the antiatherogenic effects of NO and the ratio of NO to superoxide anion may be reduced in atherosclerotic arteries, leading to very rapid reaction of NO with superoxide anion and production of highly reactive peroxynitrite, which can directly damage lipids and protein.

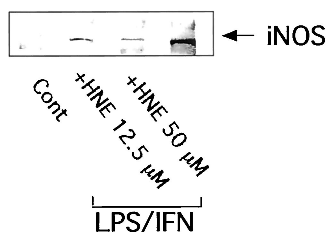


**Figure 6.** Effect of HNE on iNOS protein expression in rat VSMCs. Western blot analysis of iNOS protein expression. Cells were treated with LPS/IFN in the presence of various concentrations of HNE for 24 hours. After treatment, iNOS protein was detected as a band with a molecular mass of ≈125 kDa.

It has been reported that HNE induced relaxation of human cerebral arteries[31] and mesenteric arteries[32] in an endothelium-dependent manner. HNE could induce intracellular peroxide production[33] in endothelial cells, which may be a mediator of endothelium-dependent relaxation.[34] It should be evaluated whether HNE causes endothelial cell NOS dysfunction, which could trigger the compensatory response (superoxide anion generation followed by hydrogen peroxide production), possibly representing an important mechanism underlying oxidative vascular injury.[34] HNE treatment of the cells resulted in depletion of intracellular glutathione (GSH).[33] GSH plays an important role in the maintenance of the intracellular redox balance and the regulation of several redox-sensitive transcription factors, such as NF-κB. Because the ability of LPS or cytokines to induce NF-κB activation has been shown to be dependent on the generation of cellular redox stress, decreased GSH levels could lead to enhanced response of NF-κB activation, thereby increasing iNOS induction. Although activation signals initiating protein kinase signaling cascades that result in the phosphorylation of IκB on degradation by the proteosome could be boosted by depleted GSH levels with HNE treatment, HNE does selectively block signaling events that are required for IκB-α degradation. Therefore, it is unlikely that HNE permits NF-κB to translocate to the nucleus and bind its recognition elements within the promoter regions of NF-κB responsive genes, including iNOS.

It has been reported that HNE was found to be toxic to cells at high concentrations, whereas lower concentrations induced cell growth and DNA synthesis.[35–37] Similar effects have been observed with other oxidation products, such as $H_2O_2$ and oxidized LDL, which, over a narrow concentration range, can cause proliferative and cytotoxic effects.[38,39] HNE may exhibit concentration-dependent effects, modulating the expression of different genes at different concentrations. This may be relevant in atherogenesis, in which cell growth, apoptosis, and necrosis are thought to contribute equally to lesion formation.

In conclusion, our data suggest that the aldehyde HNE is one of the active components in oxidized LDL responsible for the inhibitory capacities of this lipoprotein on the NF-κB system. Treatment with HNE appears to selectively block signaling events that are required for IκB-α degradation, thereby preventing NF-κB activation. Inhibition of NF-κB–regulated gene expression, especially modulation of NO production, may contribute to certain stages of atherogenesis.

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## References

1. Yla-Herttuala S, Palinski W, Rosenfeld ME, Parthasarathy S, Carew TE, Butler S, Witztum JL, Steinberg D. Evidence for the presence of oxida-

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tively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J Clin Invest*. 1989;84:1086–1095.

2. Palinski W, Rosenfeld ME, Yla-Herttuala S, Gurtner GC, Socher SS, Butler SW, Parthasarathy S, Carew TE, Steinberg D, Witztum JL. Low density lipoprotein undergoes oxidative modification in vivo. *Proc Natl Acad Sci U S A*. 1989;86:1372–1376.
3. Esterbauer H, Jurgens G, Quehenberger O, Koller E. Autoxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. *J Lipid Res*. 1987;28:495–509.
4. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med*. 1991;11:81–128.
5. Berliner JA, Navab M, Fogelman AM, Frank JS, Demer LL, Edwards PA, Watson AD, Lusis AJ. Atherosclerosis: basic mechanisms: oxidation, inflammation, and genetics. *Circulation*. 1995;91:2488–2496.
6. Brand K, Page S, Rogler G, Bartsch A, Brandl R, Knuechel R, Page M, Kaltschmidt C, Baeuerle PA, Neumeier D. Activated transcription factor nuclear factor-kappa B is present in the atherosclerotic lesion. *J Clin Invest*. 1996;97:1715–1722.
7. Baeuerle PA, Baltimore D. NF-kappa B: ten years after. *Cell*. 1996;87: 13–20.
8. Sherman MP, Aeberhard EE, Wong VZ, Griscavage JM, Ignarro LJ. Pyrrolidine dithiocarbamate inhibits induction of nitric oxide synthase activity in rat alveolar macrophages. *Biochem Biophys Res Commun*. 1993;191:1301–1308.
9. Mulsch A, Schray-Utz B, Mordvintcev PI, Hauschildt S, Busse R. Diethyldithiocarbamate inhibits induction of macrophage NO synthase. *FEBS Lett*. 1993;321:215–218.
10. Xie Q, Kashiwabara Y, Nathan C. Role of transcription factor NF-kB/Rel in induction of nitric oxide synthase. *J Biol Chem*. 1994;269:4705–4708.
11. Griscavage JM, Wilk S, Ignarro LJ. Inhibitors of the proteasome pathway interfere with induction of nitric oxide synthase in macrophages by blocking activation of transcription factor NF-kappa B. *Proc Natl Acad Sci U S A*. 1996;93:3308–3312.
12. Nathan C. Nitric oxide as a secretory product of mammalian cells. *FASEB J*. 1992;6:3051–3064.
13. Lowenstein CJ, Snyder SH. Nitric oxide, a novel biologic messenger. *Cell*. 1992;70:705–707.
14. Moncada S, Higgs EA. Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB J*. 1995;9:1319–1330.
15. Napoli C, D'Armiento FP, Mancini FP, Postiglione A, Witztum JL, Palumbo G, Palinski W. Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal hypercholesterolemia: intimal accumulation of low density lipoprotein and its oxidation precede monocyte recruitment into early atherosclerotic lesions. *J Clin Invest*. 1997;100:2680–2690.
16. Muller K, Hardwick SJ, Marchant CE, Law NS, Waeg G, Esterbauer H, Carpentar KL, Mitchinson MJ. Cytotoxic and chemotactic potencies of several aldehydic components of oxidised low density lipoprotein for human monocyte-macrophages. *FEBS Lett*. 1996;388:165–168.
17. Leonarduzzi G, Scavazza A, Biasi F, Chiarpotto E, Camandola S, Vogel S, Dargel R, Poli G. The lipid peroxidation end product 4-hydroxy-2,3-nonenal up-regulates transforming growth factor beta1 expression in the macrophage lineage: a link between oxidative injury and fibrosclerosis. *FASEB J*. 1997;11:851–857.
18. Camandola S, Scavazza A, Leonarduzzi G, Biasi F, Chiarpotto E, Azzi A, Poli G. Biogenic 4-hydroxy-2-nonenal activates transcription factor AP-1 but not NF-kappa B in cells of the macrophage lineage. *Biofactors*. 1997;6:173–179.
19. Blanc EM, Kelly JF, Mark RJ, Waeg G, Mattson MP. 4-Hydroxynonenal, an aldehydic product of lipid peroxidation, impairs signal transduction associated with muscarinic acetylcholine and metabotropic glutamate receptors: possible action on G alpha(q/11). *J Neurochem*. 1997;69: 570–580.
20. Page S, Fischer C, Baumgartner B, Haas M, Kreusel U, Loidl G, Hayn M, Ziegler-Heitbrock HW, Neumeier D, Brand K. 4-Hydroxynonenal prevents NF-kappaB activation and tumor necrosis factor expression by inhibiting IkappaB phosphorylation and subsequent proteolysis. *J Biol Chem*. 1999;274:11611–11618.
21. Chamley-Campbell J, Campbell GR, Ross R. The smooth muscle cell in culture. *Physiol Rev*. 1979;59:1–61.
22. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*. 1987;162:156–159.
23. Gross SS, Levi R. Tetrahydrobiopterin synthesis: an absolute requirement for cytokine induced nitric oxide generation by vascular smooth muscle. *J Biol Chem*. 1992;267:25722–25729.
24. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983;65:55–63.

24a. Nunokawa Y, Ishida N, Tanaka S. Cloning of inducible nitric oxide synthase in rat vascular smooth muscle cells. *Biochem Biophys Res Commun*. 1993;191:89–94.

25. Hattori Y, Hattori S, Kasai K. Troglitazone upregulates nitric oxide synthesis in vascular smooth muscle cells. *Hypertension*. 1999;33: 943–948.
26. Smith J, Gross SS. Cloning and regulation of the rat GTP cyclohydrolase gene: coordinate transcriptional induction with iNOS by immunostimulants. In: Moncada S, Stamler J, Gross S, Higgs EA, eds. *The Biology of Nitric Oxide, Part 5*. London, UK: Portland Press; 1996:8.
27. Hattori Y, Banba N, Gross SS, Kasai K. Glycated serum albumin-induced nitric oxide production in vascular smooth muscle cells by nuclear factor κB-dependent transcriptional activation of inducible nitric oxide synthase. *Biochem Biophys Res Commun*. 1999;259:128–132.
28. Thanos D, Maniatis T. NF-kappa B: a lesson in family values. *Cell*. 1995;80:529–532.
29. Verbeuren TJ, Jordaens FH, Zonnekeyn LL, Van Hove CE, Coene MC, Herman AG. Effect of hypercholesterolemia on vascular reactivity in the rabbit, I: endothelium-dependent and endothelium-independent contractions and relaxations in isolated arteries of control and hypercholesterolemic rabbits. *Circ Res*. 1986;58:552–564.
30. Ludmer PL, Selwyn AP, Shook TL, Wayne RR, Mudge GH, Alexander RW, Ganz P. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *N Engl J Med*. 1986;315:1046–1051.
31. Martinez MC, Bosch-Morell F, Raya A, Roma J, Aldasoro M, Vila J, Lluch S, Romero FJ. 4-Hydroxynonenal, a lipid peroxidation product, induces relaxation of human cerebral arteries. *J Cereb Blood Flow Metab*. 1994;14:693–696.
32. Romero FJ, Romero MJ, Bosch-Morell F, Martinez MC, Medina P, Lluch S. 4-Hydroxynonenal-induced relaxation of human mesenteric arteries. *Free Radic Biol Med*. 1997;23:521–523.
33. Uchida K, Shiraishi M, Naito Y, Torii Y, Nakamura Y, Osawa T. Activation of stress signaling pathways by the end product of lipid peroxidation: 4-hydroxy-2-nonenal is a potential inducer of intracellular peroxide production. *J Biol Chem*. 1999;274:2234–2242.
34. Cosentino F, Katusic ZS. Tetrahydrobiopterin and dysfunction of endothelial nitric oxide synthase in coronary arteries. *Circulation*. 1995;91: 139–144.
35. Krokan H, Grafstrom RC, Sundqvist K, Esterbauer H, Harris CC. Cytotoxicity, thiol depletion and inhibition of O6-methylguanine-DNA methyltransferase by various aldehydes in cultured human bronchial fibroblasts. *Carcinogenesis*. 1985;6:1755–1759.
36. Tessitore L, Matera L, Bonelli G, Baccino FM, Dianzani MU. Aliphatic aldehydes inhibit the proliferative response of human peripheral blood lymphocytes to phytohemagglutinin and alloantigens. *Chem Biol Interact*. 1987;62:217–226.
37. Ruef J, Rao GN, Li F, Bode C, Patterson C, Bhatnagar A, Runge MS. Induction of rat aortic smooth muscle cell growth by the lipid peroxidation product 4-hydroxy-2-nonenal. *Circulation*. 1998;97:1071–1078.
38. Chatterjee S. Role of oxidized human plasma low density lipoproteins in atherosclerosis: effects on smooth muscle cell proliferation. *Mol Cell Biochem*. 1992;111:143–147.
39. Guyton KZ, Liu Y, Gorospe M, Xu Q, Holbrook NJ. Activation of mitogen-activated protein kinase by $H_2O_2$: role in cell survival following oxidant injury. *J Biol Chem*. 1996;271:4138–4142.

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