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Glycated Serum Albumin-Induced Nitric Oxide Production in Vascular Smooth Muscle Cells by Nuclear Factor κB-Dependent Transcriptional Activation of Inducible Nitric Oxide Synthase

Yoshiyuki Hattori,*[,1] Nobuyuki Banba,* Steven S. Gross,† and Kikuo Kasai*
**Department of Endocrinology, Dokkyo University School of Medicine, Mibu, Japan; and*
†Department of Pharmacology, Cornell University Medical College, New York, New York

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Glycated proteins, including serum albumin, may be involved in the pathogenesis of diabetic vasculopathy. Recent evidence suggests that expression of inducible nitric oxide synthase (iNOS) in vascular smooth muscle cells (VSMC) may, in part, promote atherosclerosis by increasing local oxidative stress. We therefore investigated whether VSMC exposed to glycated human serum albumin (GHSA) produce nitric oxide (NO) by increasing iNOS expression through transcriptional activation of the iNOS gene and whether this process is dependent on nuclear factor κB (NF-κB) activation. Treatment of VSMC with GHSA causes activation of NF-κB and the iNOS promoter. Induction of NF-κB and the iNOS promoter by GHSA exhibited dose-dependent kinetics at concentrations ranging from 3 to 1000 μg/ml. GHSA alone was a weak inducer of NO production in VSMC as measured by determining nitrite levels, and interferon-γ alone was totally ineffective, whereas the combination of GHSA and interferon-γ was a strong stimulus. This synergy for NO production corresponded to Northern blot analyses of iNOS mRNA expression. Thus, GHSA may promote atherosclerosis in part by activation of NF-κB and up-regulation of iNOS, thereby fostering local inflammation and oxidative stress. © 1999 Academic Press

Hyperglycemia is generally believed to be a major contributing factor leading to the development of diabetic complications. As blood glucose concentrations rise, excessive nonenzymatic glycation of a wide range of proteins, including serum albumin, occurs (1). Production of glycation adducts may result in a series of pathophysiologic consequences (2-4). Once advanced glycation end-products (AGEs) are formed, this process and its associated tissue damage become irreversible.

Nonenzymatic glycation is a condensation reaction between glucose and reactive protein amino acid groups, yielding Schiff base intermediates that undergo Amadori rearrangement to form stable protein-glucose adducts (5-7). Amadori adducts are the prominent form of circulating glycated proteins *in vivo*, and their concentration is significantly increased in diabetic during hyperglycemia (5-7). It has recently been demonstrated that nonenzymatically glycated serum albumin impairs the proliferation index and increases the synthesis and gene expression of type IV collagen in mesangial cells (8). Moreover, mesangial expansion observed in a rodent model of genetic diabetes was significantly attenuated by injecting an antibody selectively reacting with Amadori adducts (9-10). In murine vascular endothelial cells, glycated albumin was shown to enhance nitric oxide (NO) synthase activity and gene expression (11). Glycated serum albumin has also been shown to induce chemokine gene expression in human retinal pigment epithelial cells and corneal keratinocytes (12,13). Although these data may support the role of glycated proteins in the pathogenesis of diabetic complications, the mechanisms by which early glycosylated products of albumin exert these effects are still unknown.

Accelerated atherosclerosis in patients with diabetes is a major cause of their morbidity and mortality. Recent evidence indicates that vascular smooth muscle cells (VSMC) can express the inducible isoform of NO synthase (iNOS). The putative role of iNOS in the development and clinical expression of atherosclerotic vascular disease is not yet clear, although some investigators have speculated that iNOS activity may, in part, be responsible for oxidative damage to VSMC. For these reasons, we sought to define the effects of glycated human serum albumin (GHSA) on iNOS expres-

[1] Corresponding author. Fax: + 81 282 (86) 4632. E-mail: yhattori@dokkyomed.ac.jp.

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sion in VSMC. We investigated whether GHSA induces NO formation by rat VSMC with an emphasis on the nuclear factor κB (NF-κB) activation and iNOS promoter activation followed by iNOS gene expression.

MATERIALS AND METHODS

Cell culture and RNA extraction. VSMC were isolated by elastase and collagenase digestion of thoracic aortae from male Wistar rats, as previously described (14). Cells in passages 10 to 15 were used for experiments. Total RNA was extracted from confluent VSM using a modified guanidinium isothiocyanate method (15).

Nitrite assay. Nitrite accumulation, an indicator of NO synthesis, was measured in the cell culture medium of confluent VSMC (16). Nitrite was quantified colourimetrically after adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid) to 100-μl samples. Absorbance at 550 nm was determined with a microplate reader (Molecular Devices, Richmond, CA, USA). Nitrite concentrations were calculated by comparison with the absorbance of standard solutions of sodium nitrite prepared in cell culture medium.

Northern blot analysis of iNOS mRNA. An iNOS cDNA [kindly provided by Dr. Nunokawa (17)] labelled with [α-^{32}P]dCTP by random priming was used as a probe. Total RNA (10 μg per lane) was subjected to electrophoresis on a 1.2% agarose gel containing formaldehyde, and transferred to nitrocellulose filters. The filters were prehybridised at 68°C for 15 min and then hybridised with the ^{32}P-labelled iNOS cDNA probe in a rapid hybridisation solution (QUIKHYB; Stratagene, La Jolla, CA, USA) at 68°C for 1 h. The hybridised filters were washed twice for 15 min at room temperature with 2× SSC/0.1% SDS, and then twice for 30 min at 60°C with 0.1× SSC/0.1% SDS. The filters were exposed to an imaging plate (Fuji Photo Film Co., Tokyo, Japan) at room temperature for 6 h and analysed using a FUJIX bioimaging analyser (BAS2000II, Fuji Photo Film Co.). Filters were then stripped and reprobed for the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

NF-κB activation. To study NF-κB activation, the cells were stably transfected with a cis-reporter plasmid containing the luciferase reporter gene linked to five repeats of NF-κB binding sites (pNFkB-Luc: Stratagene). For this, the pNFκB-Luc plasmid was transfected together with a pSV2neo helper plasmid (Clontech, Palo Alto, CA, USA) into rat VSMC using FuGEN 6 transfection reagent (Boehringer Mannheim, Mannheim, Germany). 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 using a cloning cylinder and analysed individually for expression of luciferase activity. Several clones were selected for analysis of NF-κB activation. Luciferase activity was measured using a Luciferase assay kit (Stratagene).

iNOS promoter analysis. iNOS promoter function was studied, as previously described (18), using rat VSMC stably transfected with a construct containing a 1.7-kb fragment of the mouse iNOS promoter which was cloned in front of a reporter gene that encodes the secreted form of human placental alkaline phosphatase (SEAP). SEAP activity, which is released into the cell culture medium, was measured by a sensitive chemiluminescent assay.

Statistical analysis. Data are presented as the mean ± SEM. Multiple comparisons were evaluated by ANOVA followed by Fisher's protected least significant difference test. Student's unpaired *t* test was used for comparisons between two experiments. A value of $P < .05$ was considered statistically significant.

Materials. Recombinant murine interferon-γ was obtained from Genzyme (Cambrigde, MA, USA). Human serum albunin (HSA, nonglycated) and glycated human serum albumin (GHSA) were obtained from Sigma (St. Louis, MO, USA). GHSA, which contains 1-5 moles of fructosamine per mole albumin, was made by Sigma as described by Baynes et al. (19). The GHSA preparation did not contain measurable AGEs as determined by fluorescence assays (from 360 to 600 nm) upon excitation at 370 nm or 350 nm. The GHSA preparation also did not contain contaminant bacterial endotoxin (LPS) when measured with ENDOSPECY, a kit for endotoxin test (Seikagakukogyo, Tokyo, Japan). The ~35 kDa extracellular domain of a rat cellular receptor for AGEs, termed soluble RAGE (sRAGE), was kindly provided by Dr. Y. Kawakami (Tsukuba University School of Medicine, Japan).

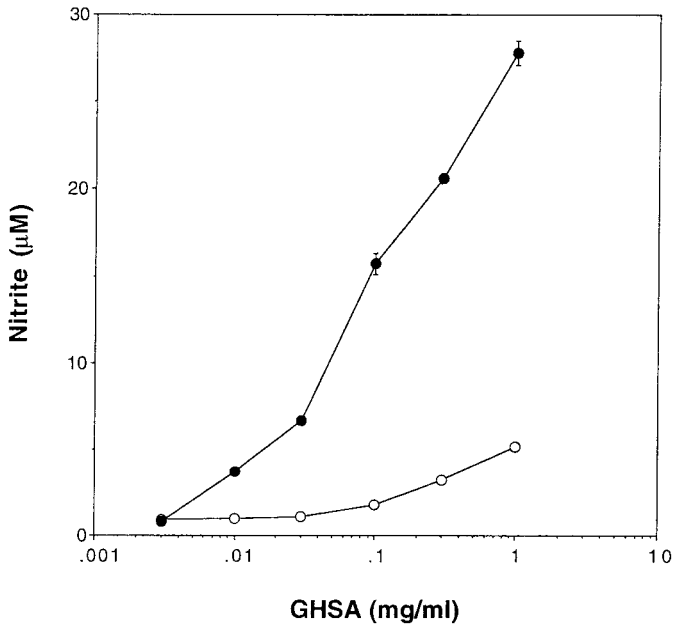

FIG. 1. GHSA and IFN synergise to increase nitrite synthesis in vascular smooth muscle cells. After treatmernt with GHSA (0.003 to 1 mg/ml, open circles) alone or in combination with IFN (100 U/ml, closed circles) for 24 h, nitrite accumulation in the medium was measured. Data are the mean ± SEM of four experiments.

RESULTS

Nitrite secretion. Unstimulated VSMC or VSMC stimulated with nonglycated HSA did not produce detectable levels of nitrite. GHSA slightly increased nitrite accumulation in the cell culture medium at higher concentrations (Figure 1). IFN alone (at concentrations up to 1000 U/ml) did not induce detectable levels of nitrite (data not shown). However, GHSA-induced nitrite release was markedly potentiated by IFN. In the presence of IFN, nitrite accumulation increased as a function of GHSA concentration (3 to 1000 μg/ml) (Figure 1). This GHSA/IFN-induced nitrite production was not reduced by polymyxin B (10 μg/ml), an LPS inhibitor (data not shown).

NF-κB activation. GHSA dose-dependently stimulated NF-κB activity in VSMC (Figure 2). In contrast, nonglycated HSA was totally ineffective (data not shown). IFN alone had no effect on NF-κB activity, but modestly enhanced GHSA-induced NF-κB activity (in-

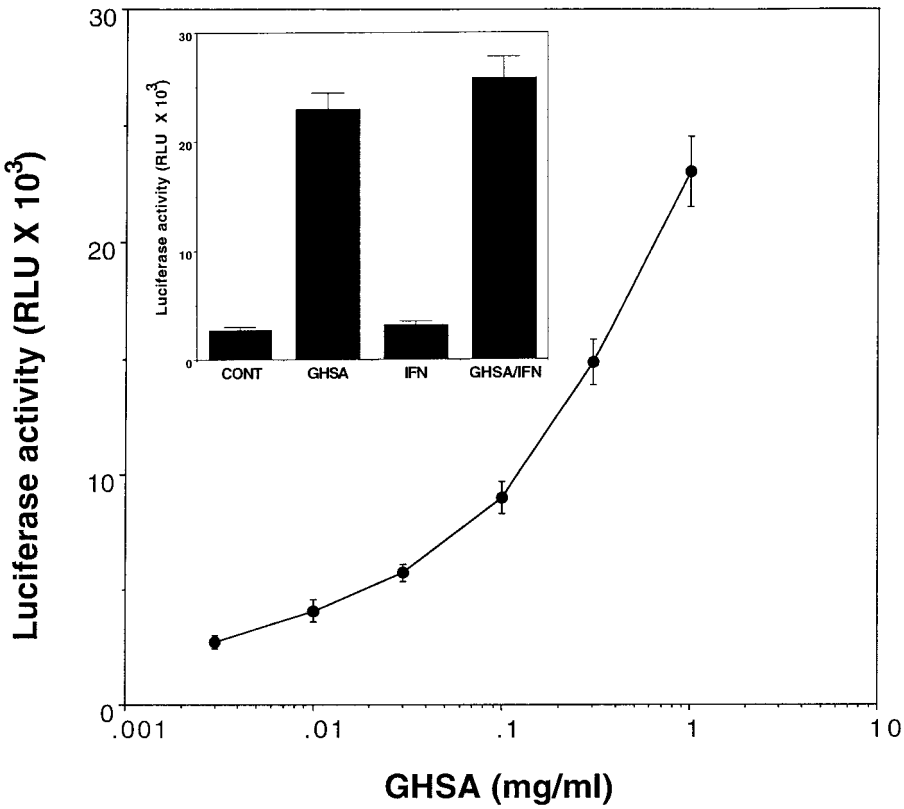

FIG. 2. The effect of GHSA on NF-κB-dependent transcriptional activity. Vascular smooth muscle cells were transfected with pNFκB-Luc (a *cis*-reporter plasmid containing the luciferase reporter gene linked to five repeats of an NF-κB binding site). The cells were treated with different concentrations of GHSA (0.003 to 1 mg/ml). After 4 h, cells were lysed, and luciferase activities were measured. Data are the mean ± SEM of triplicate observations. Inset: the cells were left untreated (CONT) or were treated with GHSA (1 mg/ml) alone, INF (100 U/ml) alone or both (GHSA/IFN) for 4 h, after which luciferase activities were measured.

set of Figure 2). NF-κB activity increased in GHSA-treated VSMC by 8.5 fold relative to unstimulated levels. NF-kB activity elicited by GHSA or in combination with IFN was not affected by an excess of sRAGE (data not shown).

iNOS promoter activation. GHSA activated the iNOS promoter in a concentration-dependent fashion in VSMC (Figure 3). IFN alone did not affect the iNOS promoter activity but enhanced GHSA-induced activity (Figure 3). iNOS promoter activity increased in VSMC treated with GHSA alone or in combination with IFN by 3.1 and 4.4 fold relative to unstimulated levels, respectively. iNOS promoter activity elicited by GHSA or in combination with IFN was not affected by an excess of sRAGE (data not shown).

iNOS mRNA expression. iNOS mRNA expression was minimal in unstimulated VSMC, as measured by Northern blot analysis (Figure 4). IFN is totally ineffective and GHSA alone is a weak inducer of iNOS mRNA, whereas the combination of GHSA and IFN is a strong stimulus for induction of iNOS mRNA (Figure 4).

DISCUSSION

This study demonstrates that GHSA stimulates NF-κB activity and induces transcription of the iNOS gene in VSMC. The induction of NO synthesis in VSMC by GHSA was remarkably synergistic with IFN. This synergy is likely to be a consequence of induced iNOS activity and is associated with an increase in iNOS mRNA levels. Regulation of iNOS is considered largely transcriptional and mediated by the activation of transcriptional factor NF-κB (20,21), while marked potentiation of iNOS activity caused by LPS or other cytokines is mediated by a mechanism which is synergistic with IFN (16,22). We showed that GHSA alone is a weak inducer of iNOS, and IFN alone is totally ineffective, whereas the combination of GHSA and IFN is a strong stimulus for induction of iNOS mRNA. The concentrations of GHSA for half-maximal stimulation of VSMC NF-κB and iNOS promoter activation were 120 and 100 μg/ml, respectively, and this value was 80 μg/ml for nitrite production in the presence of IFN. These values for half-maximal stimulation were well below the normal plasma concentration range of GHSA

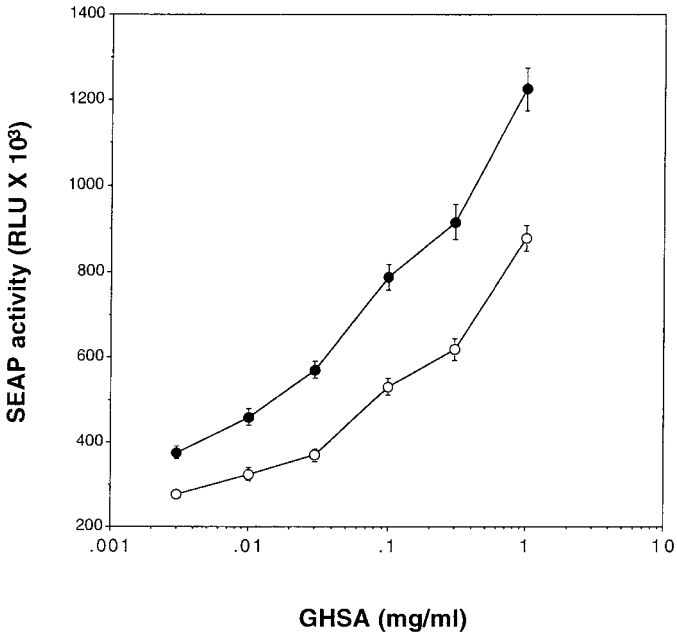

FIG. 3. Effect of GHSA on iNOS promoter/SEAP reporter expression in stably transfected rat vascular smooth muscle cells. After treatmernt with GHSA (0.003 to 1 mg/ml, open circles) alone or in combination with IFN (100 U/ml, closed circles) for 24 h, SEAP activity in the cell culture medium was measured.

(350 to 500 μg/ml) (23,24), and at concentrations exceeding the normal plasma range, GHSA potently activated VSMC. Because of the close correlation between serum glucose content and the degree of albumin glycation, the concentrations at which GHSA could induce high output NO synthesis together with a possible increase in production of cytokines such as IFN are likely to be easily reached in diabetics.

The GHSA used in our study was purified to exclude residual contamination with AGEs as described by Baynes (19). Moreover, treatment of the cells with a soluble form of the receptor for AGEs affected neither NF-κB nor iNOS promoter activity elicited by GHSA. These results indicate that the response obtained with GHSA is not due to AGEs. We did not find endotoxin contamination in our GHSA preparation using an endotoxin detection kit. We found substantial inhibition of LPS-induced nitrite production by polymyxin B, whereas polymixin did not affect the induction of nitrite elicited by GHSA together with IFN. These data strongly suggest that LPS contamination was not responsible for the responses we observed with GHSA.

NO is an important regulator of vascular function. In normal arteries, NO produced by endothelial NOS probably plays an important antiatherogenic role by causing vasorelaxation and inhibiting VSMC proliferation, platelet aggregation, and lipoprotein oxidation (25-27). Also, recent studies indicate that inhibition of NO synthesis promotes atherosclerosis, whereas supplementation with L-arginine reduces atherogenesis (28). By contrast, there is evidence that the NO-derived oxidant peroxinitrite is actively involved in atherogenesis (29-31). Peroxynitrite is a product of the reaction between NO and superoxide anion (32), and its formation often occurs at sites where there is stimulated overproduction of NO (33). In addition, an increase in iNOS mRNA expression for any level of stimulation may result in an increase in superoxide anion production by the enzyme under conditions in which L-arginine or selected cofactors are limited. Indeed, diabetes may be associated with lower availability of L-arginine and/or the NOS cofactor tetrahydrobiopterin (34,35). High output production of NO, which may favor peroxynitrite formation, is generally associated with iNOS, and it is likely that many of the inflammatory cytokines associated with atherosclerosis (36), in particular, IFN-γ, interleukin-1, and tumor necrosis factor-α, may lead to stimulated expression of iNOS.

Activation of NF-κB by GHSA may also have broader implications for atherosclerosis. Recent studies implicate the existence of an oxidant-sensitive transcriptional pathway that activates the expression of cell adhesion molecules and chemokines such as vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein 1 (MCP-1) (37,38). In animal models, a number of metabolic alterations that predispose to atherogenesis such as diabetes are associated with endothelial elaboration of superoxide anion (39). The elaboration of superoxide anion is associated with activation of the transcriptional protein NF-κB, induced expression of VCAM-1 and MCP-1, and enhanced endothelial adhesiveness for monocytes. This oxidant-sensitive transcriptional pathway may be the final common pathway by which a variety of risk factors to regulate its activation. These results also suggest a role for GHSA as an additional hyperglycemia-responsive inflammatory mediator which activates NF-κB in atherosclerosis.

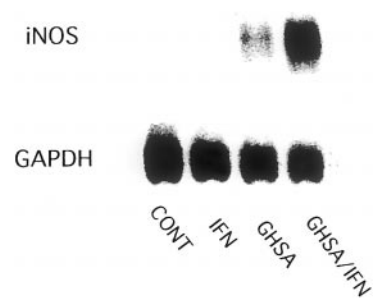

FIG. 4. GHSA and IFN synergise to cause the induction of mRNA for iNOS in vascular smooth muscle cells. Total RNA was prepared from untreated cells (CONT), cells treated with IFN (100 U/ml) alone, GHSA (1 mg/ml) alone, or both (GHSA/IFN) for 8 h. The iNOS mRNA levels were evaluated by Northern blot analysis using an iNOS specific probe and subsequently a GAPDH-specific probe.

Thus, GHSA may promote atherosclerosis in part by activation of NF-κB and upregulating iNOS, thereby fostering local inflammation and oxidative stress. Interestingly, unique binding sites for GHSA have been identified recently in plasma membranes from rat peritoneal cells. These receptor proteins have molecular weights distinct from that for the AGEs (40,41). Elucidation of GHSA receptor(s) among different cell types and the resultant postreceptor signaling will provide new insights into the molecular mechanisms underlying GHSA-mediated proinflammatory responses which may link to acceleration of diabetic complications.

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REFERENCES

1. Winterhalter, K.H. (1985) *Prog. Clin. Biol. Res.* **195,** 109–122.
2. Kennedy, L., and Baynes, J.W. (1984) *Diabetologia.* **26,** 93–98.
3. Brownlee, M., Vlassara, H., and Cerami, A. (1984) *Ann. Int. Med.* **101,** 527–537.
4. Brownlee, M., Cerami, A., and Vlassara, H. (1988) *N. Engl. J. Med.* **318,** 1315–1321.
5. Bunn, H. F., Haney, D. N., Kamin, S., Gabbay, K. H., and Gallop, P. M. (1976) *J. Clin. Invest.* **57,** 1652–1659.
6. Bunn, H. F., Gabbay, K. H., and Gallop, P. M. (1978) *Science* **200,** 21–27.
7. Higgins, P. J., and Bunn, H. F. (1981) *J. Biol. Chem.* **256,** 5204–5208.
8. Cohen, M. P., and Ziyadeh, F. N. (1994) *Kidney Int.* **45,** 475–484.
9. Cohen, M. P., and Hud, E. (1989) *J. Immunol. Methods* **122,** 279–283.
10. Cohen, M. P., Hud, E., and Wu, V. Y. (1994) *Kidney Int.* **45,** 1673–1679.
11. Amore, A., Cirina, P., Mitola, S., Peruzzi, L., Gianoglio, B., Rabbone, I., Sacchetti, C., Cerutti, F., Grillo, C., and Coppo, R. (1997) *Kidney Int.* **51,** 27–35.
12. Bian, Z. M., Elner, S. G., Strieter, R. M., Glass, M. B., Lukacs, N. W., Kunkel, S. L., and Elner, V. W. (1996) *J. Leuk. Biol.* **60,** 405–414.
13. Bian, Z. M., Elner, V. M., Lukacs, N. W., Strieter, R. M., Kunkel, S. L., and Elner, S. G. (1998) *Current Eye Res.* **17,** 65–72.
14. Chamley-Campbell, J, Campbell, G. R., and Ross, R. (1979) *Physiol. Rev.* **59,** 1–61.
15. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
16. Gross, S. S., and Levi, R. (1992) *J. Biol. Chem.* **267,** 25722–25729.
17. Nunokawa, Y., Ishida, N., and Tanaka, S. (1993) *Biochem. Biophys. Res. Commun.* **191,** 89–94.
18. Smith, J., and Gross, S. S. (1996) *in* The Biology of Nitric Oxide, Part 5 (Moncada, S., Stamler, J., Gross, S., and Higgs, E.A., Eds.), pp. 8, Portland Press, London.
19. Bynes, J. W., Thorpe, S. R., and Murtiashaw, M. H. (1984) *Methods Enzymol.* **106,** 88–98.
20. Xie, Q., Whisnant, R., and Nathan, C. (1993) *J. Exp. Med.* **177,** 1779–1784.
21. Xie, Q., Kashiwabara, Y., and Nathan, C. (1994) *J. Biol. Chem.* **269,** 4705–4708.
22. Hattori, Y., and Gross, S. S. (1995) *Biochem. Mol. Biol. Int.* **37,** 439–445.
23. Negoro, H., Morley, J. E., and Rosenthal, M. J. (1988) *Am. J. Med.* **85,** 360–364.
24. Peters, T. Jr. (1985) *Adv. Prot. Chem.* **37,** 161–245.
25. Nathan, C. (1992) *FASEB J.* **6,** 3051–3064.
26. Lowenstein, C. J., and Snyder, S. H. (1992) *Cell* **70,** 705–707.
27. Moncada, S., and Higgs, E. A. (1995) *FASEB J.* **9,** 1319–1330.
28. Cooke, J. P., and Dzau, V. J. (1997) *Ann. Rev. Med.* **48,** 489–509.
29. Darley-Usmar, V. M., Hogg, N., O'Leary, V. J., Wilson, M. T., and Moncada, S. (1992) *Free Radical Res. Commun.* **17,** 9–20.
30. Graham, A., Hogg, N., Kalyanaraman, B., O'Leary, V., Darley-Usmar, V., and Moncada, S. (1993) *FEBS Lett.* **330,** 181–185.
31. White, C. R., Brock, T. A., Chang, L. Y., Crapo, J., Briscoe, P., Ku, D., Bradley, W. A., Gianturco, S. H., Gore, J., Freeman, B. A., and Tarpey, M. M. (1994) *Proc. Nat. Acad. Sci. U.S.A.* **91,** 1044–1048.
32. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **86,** 1620–1624.
33. Ischiropoulos, H., Zhu, L., and Beckman, J. (1992) *Arch. Biochem. Biophys.* **298,** 446–451.
34. Pieper, G. M. (1998) *Hypertension* **31,** 1047–1060.
35. Pieper, G. M. (1997) *J. Cardiovasc. Pharmacol.* **29,** 8–15.
36. Libby, P., and Hansson, G. K. (1991) *Lab. Invest.* **64,** 5–15.
37. Marui, N., Offermann, M. K., Swerlick, R., Kunsch, C., Rosen, C. A., Ahmad, M., Alexander, R. W., and Medford, R. M. (1993) *J. Clin. Invest.* **92,** 1866–1874.
38. Zeiher, A. M., Fisslthaler, B., Schray-Utz, B., and Busse, R. (1995) *Cir. Res.* **76,** 980–986.
39. Giugliano, D., Ceriello, A., and Paolisso, G. (1996) *Diabetes Care* **19,** 257–267.
40. Krantz, S., Brandt, R., and Gromoll, B. (1993) *Biochim. Biophys. Acta* **1177,** 15–24.
41. Salazar, R., Brandt, R., and Krantz, S. (1995) *Biochim. Biophys. Acta* **1266,** 57–63.