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Lipopolysaccharide Activates Endothelial Nitric Oxide Synthase through Protein Tyrosine Kinase

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Vascular endothelial cell injury or activation by lipopolysaccharide (LPS) plays an important role in the pathogenesis of endotoxin shock. However, the effect of LPS on NO production from vascular endothelial cells (ECs) is incompletely understood. In this study, bovine coronary venular ECs were treated with LPS and the release of NO and expression of the endothelial NO synthase (ecNOS) were examined. We found that the ecNOS activity is transiently enhanced by LPS within the time scale of about 10 h due to the interplay between two LPS-induced mechanisms. Within the first 10 h of LPS treatment, the specific activity of ecNOS is increased by a post-translational modification mediated through a protein tyrosine kinase cascade. After about 10 h of treatment, however, LPS destabilizes the transcript of ecNOS and thus decreased the expression level and total activity.

Lipopolysaccharide (LPS) has been implicated to be a causal agent of sepsis. Although sepsis-induced systemic hypotension has been linked to overproduction of nitric oxide (NO) derived from an inducible (or inflammatory) form of nitric oxide synthase (iNOS) expressed in vascular smooth muscle cells and macrophages (1-3), the effect of LPS on NO release from endothelial cells (ECs) is still unclear. It has been reported that LPS inhibits endothelium-dependent relaxation (4), attenuates the release of NO from cultured endothelial cells (5) and isolated blood vessels (4), and enhances the degradation of endothelial constitutive nitric oxide synthase (ecNOS) transcript (6). On the other hand, several lines of evidence indicate that synthesis of constitutively derived NO is increased by LPS (7,8). Salvemini et al. (7) reported that LPS enhances the release of an NO-like factor, detected by the platelet-inhibition assay, from bovine ECs. Fleming et al. (8) also found that endothelium-derived kinins increase NO production during the immediate response of ECs to LPS.

In addition to the conflicting results reported in the previous studies, the underlying mechanism for LPS-induced cellular perturbation is not known in detail. Recent studies in ECs and monocyte/macrophage have shown that LPS binding to soluble or membrane-bound CD14 stimulates the tyrosine-phosphorylation of mitogen-activated protein kinases such as ERK-1, ERK-2, and p38 (9-15), and increases IL-6 release in vascular endothelial cells (16). Melzig et al. (17)) reported that pretreatment of bovine aortic ECs with protein tyrosine kinase inhibitor, tyrphostin B46, attenuated the toxic effect of LPS. Furthermore, Adamson et al. (10) showed that protein tyrosine kinase is involved in the induction of E-selectin by LPS in human umbilical vein ECs. The involvement of protein tyrosine kinase activation in LPS-elicited cellular responses suggests that protein tyrosine kinases may interact with NOS directly or indirectly to influence NO production in ECs.

Our laboratory has demonstrated that LPS down-regulates the expression of ecNOS by decreasing the stability of ecNOS transcript (6). In an attempt to elucidate further the direct LPS effect, we hypothesized that protein tyrosine kinases are involved in the post-translational activation of ecNOS and that this event is partially responsible for the observed LPS effect on endothelial NO production. In the present study, we used bovine coronary venular endothelial cells (BCVECs) for testing this hypotheses.

MATERIALS AND METHOD

Cell culture and reagents. BCVECs were isolated from bovine hearts immediately after the death of the animal using the method described by Schelling et al. (18). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS; Summit Biotechnology, Ft. Collins, CO), 2 mM glutamine, 2 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/

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ml streptomycin, and 0.25 μg/ml amphotericin B at 37°C in 7% CO_2 under humidified atmosphere. The supplemented DMEM described above is termed complete DMEM (CDMEM) in the following. Cells from the 17th-23th passage at 5 days after reaching confluence were used in this study, unless stated otherwise. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) except indicated otherwise. When passing, the cells were mobilized with 0.25% trypsin (Gibco Laboratories, Grand Island, NY) in Dulbecco's phosphate buffered saline (DPBS) with 0.018% ethylenediaminetetraacetic acid (EDTA) and plated onto 60 mm sterile plastic tissue culture plates coated with 1.5 (w/v)% gelatin in DPBS.

One day before experiments, the medium was replaced by RPMI 1640 Select-Amine (Gibco) containing 4% FBS, 10 mM HEPES, 200 μM glutamine and 100 μM L-arginine. For the investigation of the LPS effect on NO release, cells were washed twice with DPBS, and then incubated in RPMI 1640 with and without 1 μg/ml LPS (*Escherichia coli* serotype 0111:B4) for 0, 1, 6 and 12 h.

Markers of endothelial cell viability. Cells were passaged onto 35-mm tissue culture plates containing 1 ml CDMEM. At one to four days after the cells reached confluence, LPS (1 μg/ml) was added to the medium for 6 h, and then the cells were washed twice with DPBS, trypsinized, and suspended in 0.5 ml of DPBS. The cell suspension was mixed with 0.5 ml of 0.4% trypan blue solution (w/v) and the mixture was incubated at 37°C for 7 min. The number of cells which excluded dye (live cells) and cells which stained blue (dead cells) were determined using a hemocytometer. To examine the cell morphology, cells at 2, 4, and 30 days post-confluence were treated with LPS (1 μg/ml) for 12 h and examined under phase-contrast inverted microscope (Nikon TMS) at $\times$200 magnification.

Measurement of NO. The production of NO was determined by measuring nitrite, the primary reaction product of NO in hemoglobin-free medium. Nitrate production in our sample was shown to be less than 5%. The supernatant of cell culture was collected for analysis of NO by chemiluminescence. The sample (100 μl) was injected into a reflux chamber containing glacial acetic acid-1% potassium iodide at room temperature. Under these conditions, nitrite is quantitatively converted to NO. The NO gas was then purged into a NO analyzer (Sievers Research, Inc., Boulder, CO) and quantitated by reference to $NaNO_2$ standards. The amount of NO release was normalized to the protein concentration determined by the Bradford, Coomassie brilliant blue method as described by Bio-Rad (Hercules, CA). Bovine serum albumin was used as the standard.

Measurement of agonist-stimulated NO release. Differential rate of NO release from ecNOS was measured in the presence of calcium ionophore A23178 or bradykinin (BK). Cells were incubated in RPMI 1640 Select-Amine as described above with or without 1 μg/ml LPS for 0, 1, 6 and 12 h. At the end of incubation, 10 μM A23187 or BK was added to the culture. The supernatant (100 μl) was taken for nitrite measurement before and 1 h after the addition of A23187 or BK. The difference between these two nitrite measurements, after subtracting the background level of medium for control cells (i.e. without A23187 or BK) and normalized by the total protein amount, was taken as an indication of ecNOS activity in ECs.

Western blot analysis. Protein extraction was conducted using 0.1% sodium dodecylsulfate (SDS) containing 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin and 0.1 mM PMSF. Equal quantity of proteins (10 μg/lane) were separated by electrophoresis on 7% SDS-polyacrylamide gels. The separated proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA), which was then treated with 5% dry milk in Tris-buffered saline solution pH 7.6 containing 0.1% Tween. The membrane was then incubated with a 1:2000 dilution of a rabbit polyclonal antibody (Transduction Laboratory, Lexington, KT) raised against a 20.4 kDa protein fragment corresponding to amino acids 1030-1209 of human ecNOS. The membrane was then incubated with a 1:2500 dilution of a donkey anti-rabbit immunoglobulin secondary antibody conjugated with a horse radish peroxidase (Amersham, Arlington Heights, IL). Signals were detected using the ECL detection system (Amersham) and autoradiography film.

Data analysis. Results are given as the mean $\pm$ SEM. Data analysis was performed by one-way analysis of variance followed by Fisher's PLSD test using StatView 4 (Abacus Concepts, Berkeley, CA.). Differences were considered to be significant at p-value < 0.05.

RESULTS

LPS transiently increases ecNOS activity. Since LPS is known to cause damage to ECs, the NO release would be affected by the number of cells damaged. We thus began by characterizing the LPS effect on cell viability. BCVECs were passaged onto 35 mm tissue culture plates containing 1 ml CDMEM. At one to four days after the cells reached confluence, LPS (1 μg/ml) was added to the medium for 6 h, and the cell viability was quantified by trypan blue exclusion. Results (Fig. 1) show that the viability of the cells after LPS treatment reached a minimum at about 2 days after confluence, and increased as the culture time increased. These data suggest that the survival of ECs during the insult elicited by LPS is strongly dependent on the growth stage of the cells. In general, growth-arrested cells were less sensitive to LPS. The differential LPS sensitivity was also visualized by the changes in cell morphology from cobblestone-like to retracted and rounded in shape during LPS treatment (data not shown).

To minimize the effect of cell damage and to mimic the growth-arrested state as observed in vivo, BCVECs at 5 days after confluence were used to examine the effect of LPS on NO release. To examine specifically the effect of LPS on ecNOS activity, calcium ionophore A23187 was used to increase the intracellular calcium concentration, which specifically activates ecNOS and allows the measurement of its differential NO produc-

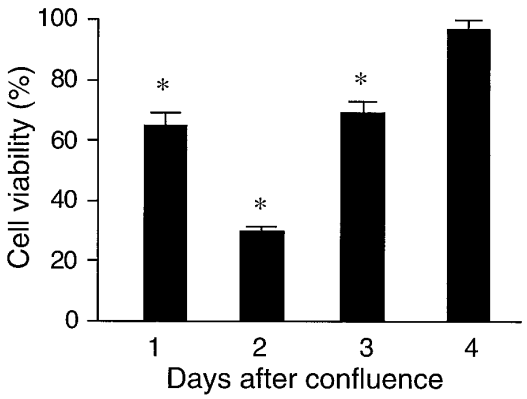

FIG. 1. Changes of cell viability during culture after incubation with LPS. One to four days after bovine coronary venular endothelial cells (BCVECs) reaching confluence, LPS (1 μg/ml) was added to the medium for 6 h, and the cell viability was quantified by trypan blue exclusion. Values are means $\pm$ SE; n = 4. *P < 0.05 vs. 4 days after confluency.

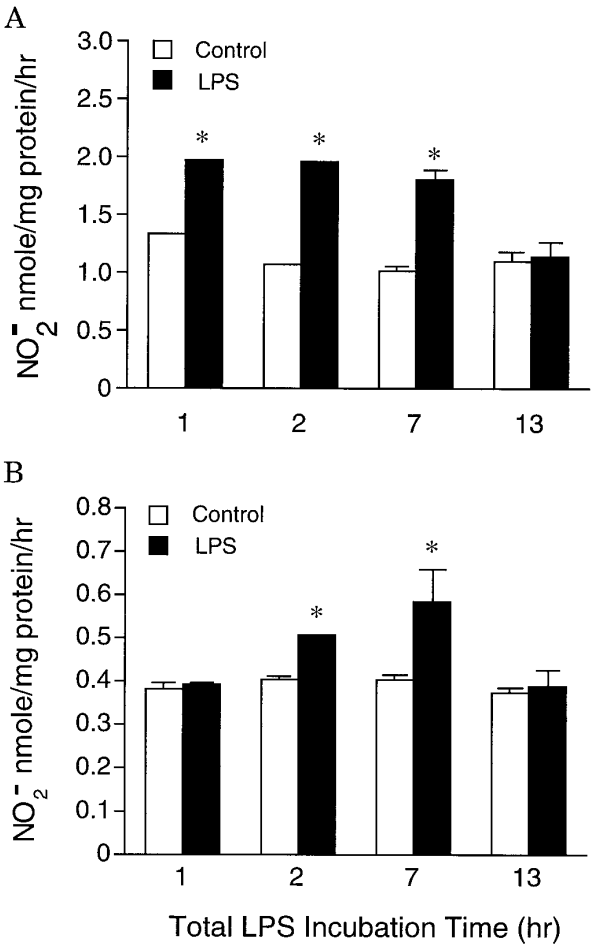

FIG. 2. LPS-induced NO release from BCVECs in the presence of calcium ionophore A23187 (A) or bradykinin (BK) (B). BCVECs were treated with or without LPS (1 μg/ml) for 0, 1, 6, and 12 h. The treated cells were washed twice with DPBS, then incubated in fresh medium with LPS (1 μg/ml) and A23187 (10 μM) (A) or BK (10 μM) (B) for another 1 h. Culture media before and 1 h after the addition of A23187 or BK were taken for nitrite measurement. Values are means ± SE; n=2 for (A); n = 5 for (B). *P $<$ 0.05 vs. control.

tion rate in intact cells. In a parallel experiment as a comparison, we also used BK (10 μM), which increases intracellular calcium concentration through a receptor-mediated mechanisms. Figures 2A and 2B show that LPS caused a transient increase in ecNOS activity. When the ecNOS activity was measured in the presence of A23187, the LPS-stimulated ecNOS activation was already significant at 1 h after the onset of LPS treatment, but the effect diminished after 13 h of incubation with LPS (Fig. 2A). When the ecNOS activity was measured in the presence of BK, the LPS-induced ecNOS activation was not significant until about 2 h after the onset of the LPS treatment, and the effect also diminished after 13 h of treatment (Fig. 2B). This result demonstrates the transient nature of LPS effect on ecNOS activity. The difference between A23187 and BK stimulations may be attributed to the receptor-mediated action elicited by BK.

Protein tyrosine kinase mediates the LPS-enhanced ecNOS activity. It has been shown that LPS down-regulates the ecNOS expression by destabilizing its transcript (6). However, the results described above show that LPS causes a transient increase in the ecNOS activity within a time frame of about 10 h. We therefore hypothesized that the observed LPS effect is caused by an increased specific activity of ecNOS via a protein tyrosine kinase-dependent pathway, rather than by an increased ecNOS protein level. To examine the ecNOS expression, the amount of ecNOS protein was determined by western analysis. Figure 3 shows that ecNOS level started to decrease after 6 h of LPS treatment, and the trend continued as the incubation time increased. The time-dependent decrease of ecNOS protein was not found in cells without LPS treatment. This result confirms that the LPS-enhanced ecNOS activity in BCVECs is not due to the upregulation of ecNOS protein level.

To determine whether protein tyrosine kinase is involved in the LPS-enhanced ecNOS activity, we examined the effect of a protein tyrosine kinase inhibitor, genistein, on the ecNOS activity of LPS-treated cells stimulated by BK. Genistein (10 μg/ml) was added to the medium 2 h prior to the addition of LPS to allow sufficient time for the drug to diffuse into the cell. It was found that genistein did not affect BK-induced NO

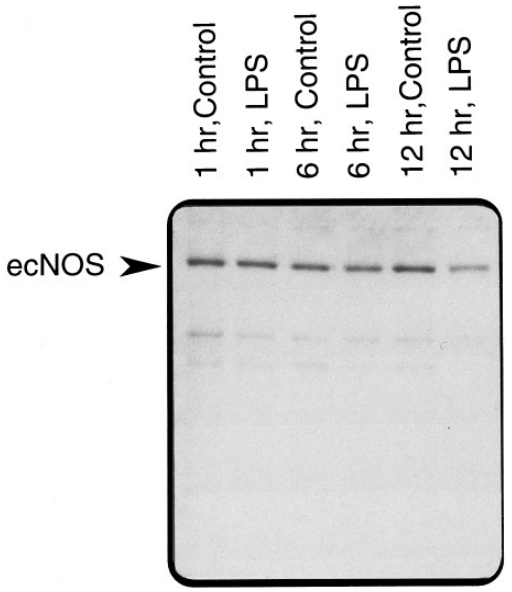

FIG. 3. Immunoblot analysis of ecNOS protein in LPS treated cells. BCVECs were treated with or without LPS (1 μg/ml) for 1, 6, and 12 h. Cell extracts (10 μg of total protein) of control and LPS-treated cells were subjected to SDS-7% polyacrylamide gel electrophoresis. Proteins were electrotransferred to polyvinylidene fluoride membrane, and the membrane was immunoblotted with a rabbit polyclonal antibody against human ecNOS.

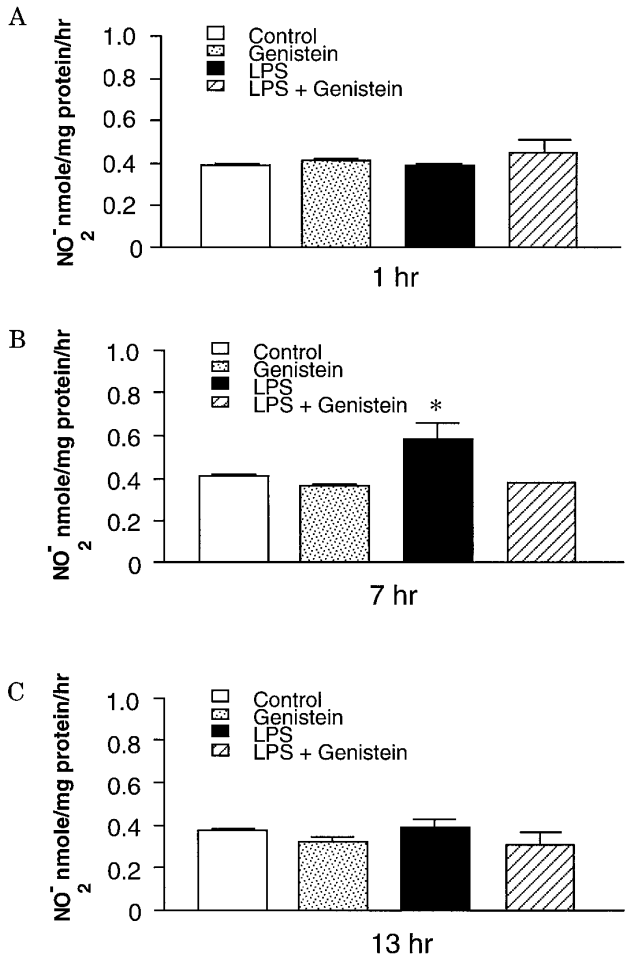

FIG. 4. Effect of genistein, a protein tyrosine kinase inhibitor, on LPS-induced NO release from BCVEC in the presence of BK. Genistein (10 μg/ml) was added to the medium 2 h prior to the addition of 1 μg/ml LPS. BK (10 μM)-activated NO release was examined at 0 (A), 6 (B), and 12 h (C). Values are means ± SE; n = 3. *P < 0.05 vs. control.

release in the absence of LPS, but it abolished the enhancement of ecNOS activity elicited by LPS (Fig. 4). This result suggests that a protein tyrosine kinase-dependent pathway mediates the enhancement of NO release exerted by LPS.

DISCUSSION

Although vascular endothelium can be activated by LPS and participates in tissue inflammation by releasing cytokines, platelet activating factor, and toxic oxygen radicals (19), the mechanism underlying the LPS effect on NO release from ECs is not understood. Previous studies have yielded inconsistent results concerning this effect (4,5,7,8,20,21) that may be due, in part, to the viability of the cell and the sequential activation of various regulatory mechanisms. In this study, we report that ecNOS activity in BCVECs is transiently enhanced by LPS within the first 10 h of treatment, and that the increased ecNOS activity is not due to the upregulation of ecNOS expression, but is attributed to a post-translational activation of ecNOS mediated by protein tyrosine kinase cascades. After about 10 h, the expression level of ecNOS starts to decrease in the presence of LPS, possibly due to decreased stability of ecNOS transcript (6). The combined effects of transcript degradation and the putative post-translational activation of ecNOS may explain the transient increase in ecNOS activity. During the first 10 h of LPS treatment, the effect of post-translational activation dominates. However, after more than 10 h of LPS treatment, the ecNOS expression level decreases significantly and thus the total ecNOS activity is compromised.

It has been reported that ecNOS activity can be modulated by calcium-independent mechanisms such as translocation from membrane to cytosol and phosphorylation at serine and tyrosine residues (22-25). In the present study, tyrosine kinase inhibitor genistein blocks the ecNOS activation in the presence of LPS, suggesting that the activation of protein tyrosine kinase by LPS is involved in the regulation of ecNOS activity. It is noteworthy that protein tyrosine phosphorylation has been reported to mediate shear stress-induced NO production in ECs (22,26), possibly through the same mechanism that activates ecNOS during LPS challenge. In addition, Adamson et al. (10) showed that protein tyrosine kinase inhibitors down-regulate the expression of E-selectin in human umbilical vein endothelial cells by blocking downstream phosphorylation of the LPS-stimulated MAP kinase. Together, these results support the involvement of protein tyrosine kinases in the cellular response to LPS.

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REFERENCES

1. Thiemermann, C. (1994) *Adv. Pharmacol.* **28,** 45–79.
2. Evans, T., Carpenter, A., Kinderman, H., and Cohen, J. (1993) *Circ. Shock* **41,** 77–81.
3. Payen, D., Bernard, C., and Beloucif, S. (1996) *Clin. Chest Med.* **17,** 333–350.
4. Myers, P. R., Zhong, Q., Jones, J. J., Tanner, M. A., Adams, H. R., and Parker, J. L. (1995) *Am. J. Physiol.* **268,** H955–H961.
5. Myers, P. R., Wright, T. F., Tanner, M. A., and Adams, H. R. (1992) *Am. J. Physiol.* **262,** H710–H718.
6. Lu, J. L., Schmiege, L. M., Kuo, L., and Liao, J. C. (1996) *Biochem. Biophys. Res. Commun.* **225,** 1–5.
7. Salvemini, D., Korbut, R., Anggard, E., and Vane, J. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 2593–2597.
8. Fleming, I., Dambacher, T., and Busse, R. (1992) *J. Cardiovasc. Pharmacol. Suppl.* **12,** S135–S138.

9. Arditi, M., Zhou, J., Torres, M., Durden, D. L., Stins, M., and Kim, K. S. (1995) *J. Immunol.* **155,** 3994–4003.
10. Adamson, P., Tighe, M., and Pearson, J. D. (1996) *Cell Adhes. Commun.* **3,** 511–525.
11. Han, J., Lee, J., Bibbs, L., and Ulevitch, R. J. (1994) *Science* **265,** 808–811.
12. Schumann, R. R., Pfeil, D., Lamping, N., Kirschning, C., Scherzinger, G., Schlag, P. K., and Herrmann, F. (1996) *Blood* **87,** 2805–2814.
13. Dziarski, R., Jin, Y. P., and Gupta, D. (1996) *J. Infect. Dis.* **174,** 777–785.
14. Sweet, M. J., and Hume, D. A. (1996) *J. Leukoc. Biol.* **60,** 8–26.
15. Sanghera, J. S., Weinstein, S. L., Aluwalia, M., Girn, J., and Pelech, S. L. (1996) *J. Immunol.* **156,** 4457–4465.
16. Arditi, M., Zhou, J., Dorio, R., Rong, G. W., Goyert, S. M., and Kim, K. S. (1993) *Infect. Immun.* **61,** 3149–3156.
17. Melzig, M. F., and Loose, R. (1995) *Pharmazie* **50,** 558–560.
18. Schelling, M. E., Meininger, C. J., Hawker, J. R., Jr., and Granger, H. J. (1988) *Am. J. Physiol.* **254,** H1211–H1217.
19. Olson, N. C., Hellyer, P. W., and Dodam, J. R. (1995) *Br. Vet. J.* **151,** 489–522.
20. Wang, P., Ba, Z. F., and Chaudry, I. H. (1995) *Am. J. Physiol.* **269,** R988–R994.
21. Walter, R., Schaffner, A., and Schoedon, G. (1994) *Biochem. Biophys. Res. Commun.* **202,** 450–455.
22. Corson, M. A., James, N. L., Latta, S. E., Nerem, R. M., Berk, B. C., and Harrison, D. G. (1996) *Circ. Res.* **79,** 984–991.
23. Ohara, Y., Sayegh, H. S., Yamin, J. J., and Harrison, D. G. (1995) *Hypertension* **25,** 415–420.
24. Nishida, K., Harrison, D. G., Navas, J. P., Fisher, A. A., Dockery, S. P. U., Nerem, R. M., Alexander, R. W., and Murphy, T. J. (1992) *J. Clin. Invest.* **90,** 2092–2096.
25. Garcia-Cardena, G., Fan, R., Stern, D. F., Liu, J., and Sessa, W. C. (1996) *J. Biol. Chem.* **271,** 27237–27240.
26. Muller, J. M., Davis, M. J., and Chilian, W. M. (1996) *Am. J. Physiol.* **270,** H1878–H1884.