Vol. 198, No. 1, 1994
January 14, 1994

LAMINAR FLOW STIMULATES ATP- AND SHEAR STRESS-DEPENDENT NITRIC OXIDE PRODUCTION IN CULTURED BOVINE ENDOTHELIAL CELLS

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Received November 4, 1993

Based on the fact that nitric oxide (NO) production is associated with changes in intracellular cGMP levels and is selectively inhibited by N^{ω} -methyl L-arginine (L-NMA), we investigated the shear stress dependency of NO production in endothelial cells (ECs) from its cGMP responses to various shear stress loads. Cultured fetal bovine aortic ECs treated with a phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX; 1 mM), were exposed to a laminar flow of Krebs buffer solution for 5 minutes in a parallel-plate flow chamber and examined for changes in intracellular cGMP levels by radioimmunoassay using an [^{125}I] cGMP kit. Application of flow increased the cGMP levels. The increase was significant in the presence of extracellular ATP (1 μM)(control, 286.1 ± 43.6; flow, 506.5 ± 44.9 fmol/10^7 cells; $p<0.001$), but not in its absence (control, 256.6 ± 60.6; flow, 301.5 ± 91.4 fmol/10^7 cells; N.S.). The cGMP levels increased significantly as the magnitude of shear stress applied increased. Treatment of ECs with a specific inhibitor of NO production, L-NMA (200 μM), completely inhibited the flow-induced increase in cGMP, and L-arginine reversed the L-NMA-induced inhibition, indicating that the increase in cGMP was due to NO produced by the flow. The flow-induced increase in NO production was markedly suppressed when extracellular Ca^{++} was chelated by adding EGTA to the perfusate. These findings suggest that flow stimulates NO production to increase cGMP levels shear stress-dependently in ECs and that extracellular Ca^{++} and ATP modulate the effects of flow.

Blood vessels dilate their luminal diameter when blood flow increases, while they reduce the diameter when blood flow decreases (1). This phenomenon has been shown to be an adaptive response of the vessels to changes in blood flow, to maintain a constant level of shear stress on the vascular wall (2), and to be endothelium-dependent (3). A number of recent studies (4-7) have suggested that nitric oxide (NO), a potent vasodilator, released from endothelial cells (ECs) may play an essential role in this vascular diameter adjustment to flow. However, no quantitative relationship between flow rate (or shear stress) and NO production in ECs has been reported,

0006-291X/94 $5.00

probably due to the following practical difficulties inherent in the methods used: 1) Culturing ECs on microcarrier beads necessary for bioassay of NO activity prevents explicit estimation of shear stress level from the applied flow load. 2) The dilution effect of flow obscures the amount of NO released.

It is, however, established that NO produced by ECs activates guanylate cyclase and dose-dependently increases intracellular cGMP levels not only in smooth muscle cells but also in ECs themselves (8-10), and that N^{ω}-methyl L-arginine (L-NMA) is a selective inhibitor of NO production. Hence, if application of flow to ECs alters their NO production, it will be reflected by the cGMP level and such a cGMP response to flow will be completely blocked by pretreatment of ECs with L-NMA. If these responses are experimentally verified, the cGMP level can be used as an indicator of NO production induced by flow. Under this working hypothesis, we attempted to elucidate shear stress-dependency of NO production in ECs. Monolayers of fetal bovine aortic ECs cultured on cover slips were exposed to controlled levels of shear stress in a specially designed flow-chamber and examined for changes in cGMP levels in the absence or presence of L-NMA.

METHODS

***Cell culture*:** ECs were isolated from bovine fetal descending aorta by collagenase (0.1%) digestion and cultured in M199 medium containing 20% fetal bovine serum, 100 U of penicillin per ml and 100 μg of streptomycin per ml at 37 °C in an atmosphere of 95% air and 5% CO_2. When the cells became confluent, they were separated with a 0.05% trypsin-2 mM EDTA solution and subcultured at a density of 1 x 10^4 cells/ cm^2 in a new flask. The cells in each subculture were counted with a Coulter counter and the number of cumulative population doublings (CPD) was calculated. All the cells used in this study had fewer than 30 CPD. The cultured cells were confirmed to be ECs by the morphological findings, i.e., a single layer pavement arrangement characteristic of ECs, the presence of factor VIII-related antigen revealed by the fluorescent antibody technique, the production of angiotensin-I converting enzyme, and the uptake of acetylated low-density lipoproteins.

***Flow-loading experiment*:** ECs which reached confluency on a glass cover slip (4.5 x 8.5 x 0.02 cm) were incubated with M199 medium containing a phosphodiesterase inhibitor, 1 mM 3-isobutyl-1-methyl-xanthine (IBMX, Sigma) for 15 minutes to preserve the cGMP level induced by application of flow in the experiment. After the cells were washed in Krebs buffer solution (glucose 10 mM, KCl 4.8 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, NaCl 119 mM, $NaHCO_3$ 24.9 mM, $CaCl_2$ 2.5 mM) bubbled with 5% CO_2 and 95% O_2, the cover slip was fixed by negative pressure in a rectangular acrylic chamber (7.3 x 3.3 x 0.05 cm) so that the cells would face toward the inside. This was a partial modification of the chamber used by Stathopulos and Hellumus (11). The chamber had an entrance and an exit for the solution and the entrance was connected to a reservoir via a silicone tube. The flow rate was controlled by a roller pump (Atto

Corp., Tokyo) which was placed between the reservoir and the chamber. The intensity of shear stress (τ, dyne/cm^2) is calculated as follows: $\tau = 3\mu Q/4ab^2$, where μ is viscosity (0.00765 p) and Q is flow volume (ml /sec) and a and b are the width (2a=3.3 cm) and height (2b=0.05 cm) of the channel, respectively. Since the Reynolds number at the maximum flow rate was 27, the flow applied in this experiment was considered to be laminar. Since the increase in internal pressure was less than 0.5 mm Hg during the perfusion at the maximum flow rate, the effect of changes in pressure was assumed to be negligible. The entire circuit was filled with Krebs buffer solution containing no ATP or 1 μM of ATP and a cyclooxygenase inhibitor, indomethacin (10 μM) , and the solution was perfused through the chamber by a roller pump at room temperature. The perfusate was not recirculated.

Determination of cGMP levels: Right after the cells were exposed to flow with different levels of shear stress (0, 0.1, 0.3, 0.6, 1 and 2 dynes /cm^2) for 5 minutes, the cover slip was removed from the chamber and 2 ml of 6% trichloroacetic acid was added to the cells. The cells were mechanically separated with a cell scraper and centrifuged (3000 rpm, 20 min., at 4 ℃). After trichloroacetic acid in the supernatant fluid was extracted with water-saturated ether, the samples were lypholized and stored at -70℃. cGMP concentrations in the samples were determined by radioimmunoassay using a [^{125}I] cGMP kit (Amersham International Ltd., U.K.) according to the acetylation protocol. The period of incubation after addition of [^{125}I] cGMP was 20 hours. In some samples, intracellular cAMP levels were measured by radioimmunoassay using a [^{125}I] cAMP kit (Amersham). To determine whether the substance which increased cGMP levels is NO, ECs were exposed to flow in the presence of L-NMA (200 μM) (12). Furthermore, the effect of a substrate of NO, L-arginine (1 mM) (13), together with L-NMA was evaluated. To assess the role of extracellular Ca^{++} in the effect of flow on NO production, similar flow-loading experiments were performed in extracellular Ca^{++}-free solutions containing a chelator of extracellular Ca^{++}, 0.2 mM EGTA.

Statistical analysis: All data are presented as means ± SD. To compare control values with those obtained during the different interventions, Student's t test for unpaired data was used. Differences were considered to be significant when the p value was less than 0.05.

RESULTS

When Krebs buffer solution was perfused at a flow rate to yield a shear stress of 0.6 dynes/cm^2, cGMP levels showed a small increase from 256.6 ± 60.6 (n=6) to 301.5 ± 91.4 (n=6) fmol/10^7 cells, which was not statistically significant. However, in the presence of extracellular ATP (1 μM), the same shear stress increased cGMP levels markedly from 286.1 ± 43.6 (n=6) to 506.5 ± 44.9 (n=6) fmol/10^7 cells ($p<0.001$) (Fig. 1). The treatment of ECs with L-NMA (200 μM) completely inhibited the flow-induced increase in cGMP. The addition of L-arginine (1 mM) to the perfusate partially reversed the inhibitory effect of L-NMA. These results revealed the validity of our working hypothesis that the flow-induced increase in cGMP levels is due to the increase in NO production.

ECs were exposed to flow with different levels of shear stress (0, 0.1, 0.3, 0.6, 1, and 2 dynes/cm^2) in the presence of 1 μM ATP. cGMP levels increased as the intensity of shear stress

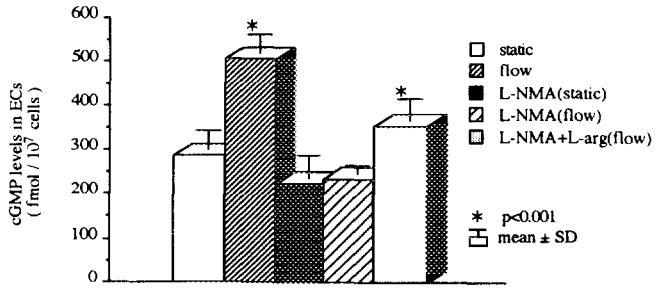

Fig. 1. Flow-induced changes in cGMP levels and the effects of L-NMA and L-arginine on them. All experiments were performed in the presence of 1μM ATP. Open column, no application of flow (static control); narrow hatched column, application of flow (with a shear stress of 0.6 dynes/cm^2) ; closed column; no application of flow, treated with 200μM L-NMA; wide hatched column, application of flow (with a shear stress of 0.6 dynes/cm^2) , treated with 200μM L-NMA; dotted column, application of flow (with a shear stress of 0.6 dynes/cm^2) , treated with 200μM L-NMA and 1mM L-arginine. These data represent means ± SD of six separate cover slips.

increased (Fig. 2). In contrast, such shear stress-dependent increases in cGMP disappeared in ECs treated with L-NMA. Unlike cGMP, intracellular cAMP levels in ECs were not changed by flow. cAMP levels at shear stresses of 0, 0.1, 0.3, 0.6, and 2 dynes/cm^2 were 28.2 ± 2.5, 34.6 ± 8.6, 28.7 ± 10.2, 40.9 ± 4.9 and 31.7 ± 8.3 pmol/10^7 cells (mean ± SD; n=3), respectively.

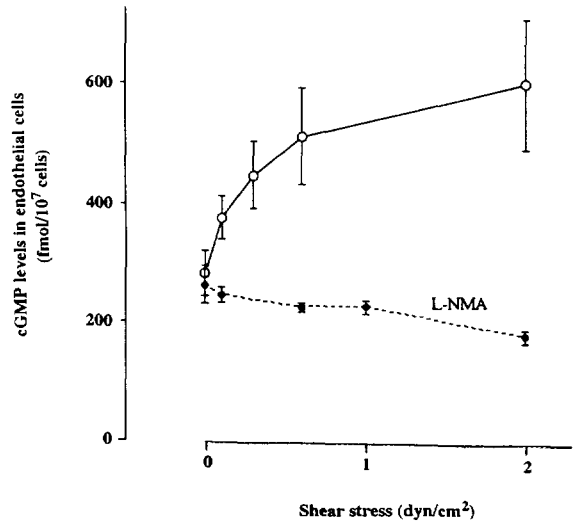

Fig. 2. Relationship between the increase in cGMP levels and the intensity of shear stress applied. Open circles show the application of flow in the presence of 1 μM ATP. Closed circles show the application of flow in the presence of 1 μM ATP and 200 μM L-NMA. Each point represents the mean ± SD of six separate cover slips.

Flow was applied to ECs in Ca^{++}-free Krebs buffer solution containing 0.2 mM EGTA and 1 μM of ATP for 5 minutes. In the absence of extracellular Ca^{++}, flow did not increase cGMP levels significantly: 271.4 ± 81.1 in the controls (n=6) vs 332.3 ± 32.2 fmol/10^7 cells at a shear stress of 0.6 dynes/cm^2 (n=6). This indicates that extracellular Ca^{++} is essential for the NO production by flow.

DISCUSSION

The present study demonstrates that flow-loading to cultured bovine fetal aortic ECs increased their intracellular cGMP levels shear stress-dependently . Many chemical stimuli including NO, norepinephrine, acetylcholine, serotonin, histamine, angiotensin II and atrial natriuretic peptide are known to increase cGMP levels in ECs (14). The flow-induced increase in cGMP levels was, however, completely blocked by L-NMA, and the inhibition was reversed by the addition of L-arginine. These findings confirmed that the increase in cGMP was due to that of NO production by flow loading. These results are consistent with the in vitro data reported by other researchers (15-17), suggesting that flow or shear stress increases the release of NO from vascular ECs. In these conventional studies, however, the characteristics of flow applied to ECs were not clearly defined and the calculated shear stresses were not adequately quantitative because ECs cultured on microcarrier beads were packed in a column perfused with solution or exposed to vortical flow induced by a magnetic stirrer in a glass chamber. The advantages of our system are that 1) steady and known levels of shear stress by laminar flow could be evenly applied to an EC monolayer on a cover slip and 2) the effect of flow on NO production could be biochemically evaluated from the changes in intracellular cGMP levels in ECs, instead of by detecting the tension of vascular rings as used in other bioassay studies.

Significant shear stress-dependency of NO production was observed only in the presence of extracellular ATP at 1 μM. In our previous study , we also demonstrated that intracellular Ca^{++} concentration ($[Ca^{++}]_i$) in ECs quantitatively corresponds to applied shear stress at the same level of ATP, and that the main source of Ca^{++} for the flow-induced increase in $[Ca^{++}]_i$ is the entry of extracellular Ca^{++} across the plasma membrane (18-20). Taking into account the fact that NO production and the activity of NO synthase are calcium-calmodulin dependent (21, 22), it seems likely that the increased Ca^{++} entry is essentially involved in the mechanism enhancing NO production in ECs. This is also supported by the fact that the removal of extracellular Ca^{++} almost eliminated the flow-induced increase in NO production. More recently, it was reported by

Ohno et al. that the flow of physiological saline solution containing no ATP elevates endothelial cGMP (23). Although the exact reason for the difference between our results and theirs is not clear, the possibility cannot be excluded that ATP released from ECs by flow (24) modulates the cGMP response because of the recirculation of solution in a dish during flow application in their flow-loading system. They observed shear stress-dependency of the cGMP response in the range from 0 to 40 dynes/cm^2, which was higher than those in other researchers' (15) and our experiments. Since extracellular ATP modulates the sensitivity of ECs to shearing force as we reported (19), ATP at around 1 μM might be necessary for ECs to increase $[Ca^{++}]_i$ sufficiently for NO production in response to such a relatively low shear stress used in this study.

It is known that the increase in cGMP levels induced by NO causes relaxation of smooth muscle cells and inhibits aggregation of platelets. Concerning the physiological significance of the increase in cGMP in ECs, recent studies have shown that the NO-induced increase in cGMP levels inhibits endothelin-1 release from ECs (25) or modulates endothelial permeability (26). Hence, NO produced in ECs by flow might modulate EC functions in an autocrine manner, probably via activation of cGMP-dependent protein kinase.

ACKNOWLEDGMENTS

This work was partly supported by Grants-in-Aid for Scientific Research and for Scientific Research on Priority Areas from the Japanese Ministry of Education, Science and Culture, a research grant for Cardiovascular Diseases from the Japanese Ministry of Health and Welfare and research funds from Tsumura & Co. and the Uehara Memorial Foundation.

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