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Cyclic Strain-Induced Plasminogen Activator Inhibitor-1 (PAI-1) Release from Endothelial Cells Involves Reactive Oxygen Species

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The molecular mechanisms of the endothelial fibrinolytic activities modulated by mechanical strain are not clear. Endothelial cells (ECs) grown on a flexible membrane base were deformed with sinusoidal negative pressures to produce an average strain of 12%. Cyclic strain induced PAI-1 release in a time-dependent manner. Strain cells resulted in a 5-fold increase in PAI-1 release. Strain induced a sustained elevated level in intracellular reactive oxygen species (ROS). Concomitantly, a sustained increase of catalase activity was observed. Both ROS and catalase activity returned to basal levels with the removal of strain. ECs pretreated with antioxidant, N-acetyl-cysteine, abolished the strain-induced ROS generation as well as strained-induced PAI-1 release. Our results indicate that cyclic strain-induced PAI-1 secretion may be mediated by an increase in ROS generation and thus emphasizes the importance of intracellular ROS in the modulation of hemodynamic force-induced cellular response of ECs.

Vascular endothelial cells (ECs) play an important role in the regulation of fibrinolytic activity in the blood by releasing various fibrinolytic substances including tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1). ECs are constantly exposed to the dynamics of mechanical forces (1, 2), including blood flow-induced shear stress and pressure-produced strain that may alter the fibrinolytic activities by modulating the secretion of tPA or PAI-1 of these affected ECs. The effects of shear stress on tPA and PAI-1 release have been previously studied (3, 4). Shear stress treatment of ECs increases tPA secretion but induces no significant changes in PAI-1 (3, 4). The effects of cyclic strain on ECs have also been recently investigated (4). In contrast to shear stress effects, cyclic strain treatment increases secretion of PAI-1, but not tPA (4). Previous studies from this laboratory have indicated that cyclic strain can stimulate the expression of endothelin-1 (Et-1) (5, 6), monocytic chemotactic protein-1 (MCP-1) (7, 8) and intercellular adhesion molecule-1 (ICAM-1) (9). However, the cellular and molecular mechanism by which mechanical deformation leads to an increased gene expression and the protein release remains largely unknown. Signal pathway including protein kinase C (6, 7) and calcium influx (6, 7, 8) have been suggested to be involved in the strain-induced Et-1 and MCP-1 expression. Recent evidence suggests that reactive oxygen species (ROS) may function as a second messenger in cells exposed to various stimuli (10, 11). ROS activation of the NF-kB and AP-1 transcription factors has been reported (12, 13). Hemodynamic forces that upregulate certain genes are believed to involve the activation of NF-κB and AP-1 (14, 15). Moreover, flow-dependent release of ROS from vascular ECs has been reported (16). However, neither the intracellular ROS levels in ECs subjected to hemodynamic forces nor the role of ROS in mechanical forces-induced cellular response have been fully defined. We have recently demonstrated that ROS is involved in the strain-induced

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Abbreviations: ECs, endothelial cells; NAC, N-acetyl-cysteine; PAI-1, plasminogen activator inhibitor-1; ROS, reactive oxygen species.

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expression of MCP-1 and ICAM-1 (17). In this study, we further demonstrate that cyclic strain can induce intracellualr ROS generation and this increased ROS level may mediate the strain-induced PAI-1 secretion. These results, thus, indicate that hemodynamic forces may exert their affects on fibrinolytic activities of ECs by modulating intracellular ROS levels.

MATERIALS AND METHODS

Materials. ECs were isolated from fresh human umbilical cords as previously described (8). Imubind PAI-1 ELISA kits were purchased from American Diagnostica Inc. (Greenwich, CT). Other chemicals of reagent grade were obtained from Sigma (St. Louis, MS).

ECs cultures. ECs were grown for 3 days in petri dishes and seeded onto the silicon membrane base of a flexcell cell plate (Flexcell, Mekeesport, PA) until the monolayer became confluent. The culture medium was changed to medium 199 containing 2% FCS, and the cells were incubated overnight before the experiments.

In vitro cyclical strain to ECs. The strain unit (FX-2000, Flexcell International Co., McKeesport, PA) consisted of a vacuum unit linked to a computerized valve control unit, described in detail elsewhere (18). The flexible membrane supporting the culture cells were deformed by a sinusoidal negative pressure with a peak level of −20 kPa which produced an average strain of 12%. After the strain treatment, the conditioned supernatant was collected for PAI-1 analysis.

Measurement of intracellular reactive oxygen species (ROS). ROS was measured by a method previously described (19, 20). In brief, strained ECs, following trypsinization, were resuspended in PBS immediately followed by incubation in 20 μM of 2′,7′-dichlorofluorescein diacetate (DCFH, Serva, Germany) at 37°C for 30 min in the dark. Reaction was stopped by centrifugation and the cell pellets resuspended in PBS. The relative fluorescence intensity of fluorophore, 2′, 7′-dichlorofluorescein (DCF), which is formed by peroxide oxidation of its nonfluorescent precursor was detected at an emission wavelength of 525 nm by using excitation wavelength of 475 nm with a Hitachi 4010 fluorescence spectrophotometer. DCFH with fresh culture medium was used as a blank control.

Measurement of catalase activity. Catalase activities were calculated as described previously (21). In brief, ECs, removed from plate and collected in PBS, were rapidly frozen at −70°C overnight. Cells were thawed, centrifuged and the supernantant containing 50 μg protein was collected. Catalase activity was determined spectrophotometrically by measuring the decomposition of exogenously added H_2O_2 (10 mM). The rate of disappearence of H_2O_2 is followed by analyzing the rate of decrease in absorbance at 240 nm. One unit is defined as 1 μmole of H_2O_2 decomposition per min at room temperature. In some experiment, cell lysate was pretreated for one half hour with specific catalase inhibitor, i.e., 3- amino-1, 2, 4-triazole (3-ATA, 20 mM) prior to catalase activity measurement.

Measurement of PAI-1 concentration. PAI-1 concentrations in culture media were assayed with Imubind-PAI-1 ELISA kits (22). Briefly, Nunc-immuno plates were pre-coated with goat anti-human PAI-1 monoclonal antibody overnight. Conditioned medium was added and incubated for 3 h. After washing, 200 μl of horseradish peroxidase-labeled goat anti-human PAI-1 was added and the reaction was developed by adding orthophenylenediamine as substrate.

RESULTS

ECs cultured on flexible membrane bases were subjected to deformation to produce an average strain of 12%. ECs under strain treatment for 24 h remained morphologically intact. The release of cellular lactate dehydrogenase did not significantly increase as compared to control unstrained cells (data not shown). PAI-1 released into culture media was measured. ECs maintained in M-199 consisting 2% serum constantly secreted PAI-1 into culture medium in a time-dependent manner (Fig. 1). PAI-1 release from unstrained control cells was 0.9 $\mu g/10^6$ cells and 1.4 $\mu g/10^6$ cells after 12 h and 24 h of incubation, respectively. PAI-1 release increased to 2.0 $\mu g/10^6$ cells and 4.3 $\mu g/10^6$ cells after 12 h and 24 h, respectively, of strain treatment. Secretion rate, as calculated from the rise in the PAI-1 concentration in the medium from unstrained control and strained cells between 24 h and 12 h, was 37 ng/h/10^6 cells and 190 ng/h/10^6 cells, respectively. Thus, 12% of strain treatment induced about a 5-fold increase in PAI-1 release. These results indicate that mechanical strain can induce PAI-1 release in ECs.

ECs under 12% treatment rapidly increased their intracellular ROS as indicated by the fluorescence intensity of the peroxide product of DCFH (Fig. 2A). This intracellular ROS level reached to a maximum by 0.5 h subsequent to strain. Elevated ROS levels then declined but still remained elevated as long as the strain was maintained. Concomitantly, a similar pattern

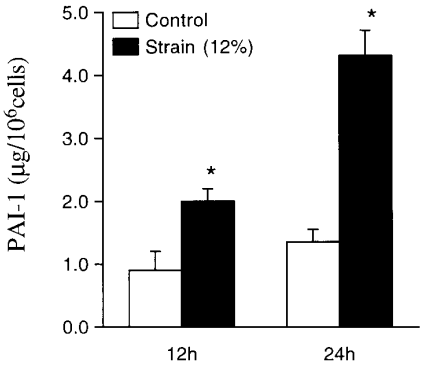

FIG. 1. Increased PAI-1 secretion from ECs after cyclic strain. PAI-1-containing culture media from control unstrained ECs or ECs under strain (12%) for 12 or 24 h were collected and analyzed by ELISA as described in Materials and Methods. All data are represented as mean±SEM from 3 to 5 experiments. *P<.05 vs control unstrained cells.

that a rapid induction followed with a sustained increase of catalase activity was observed in these strained cells (Fig. 2B). Increased ROS and catalase activity returned to the basal level subsequent to the removal of the strain. The strain-induced ROS generation and catalase activity could be abolished by pre-treating the ECs with an antioxidant, N-acetyl-cysteine (NAC). In order to confirm that the induced H_2O_2 decomposition was due to the increased

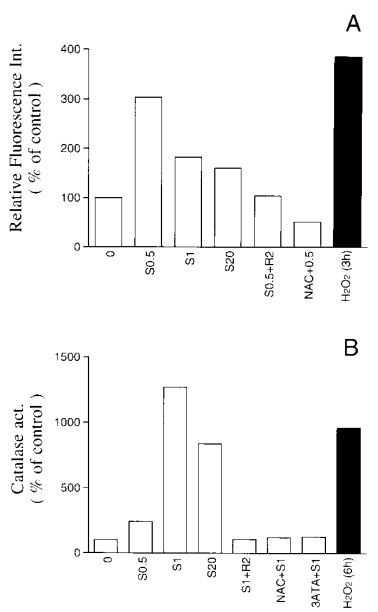

FIG. 2. Cyclic strain induces intracellular ROS and catalase activities in ECs. A) ECs under 12% strain treatment were removed from plates immediately followed by incubation with non-polar DCFH. The intracellular ROS was analyzed by measuring relative fluorescence intensity of fluorophore, DCF. B) The catalase activities were measured spectrophotometrically by the H_2O_2 decomposition rate. ECs were strained for 0.5 (S0.5), 1 (S1)or 20 h (S20). Strained ECs were allowed to rest for 2 h (S0.5+R2). ECs were pretreated with NAC (20 mM) for 0.5 h prior to strain treatment (NAC+S0.5). Cell lysates from strained cells (S1) were pretreated with 3-ATA (3ATA+S1) prior to the measurement of H_2O_2 decomposition. ECs treated with H_2O_2 (100 μM) were used as a reference for ECs under condition of oxidant stress. Representative data from 3 experiments are shown.

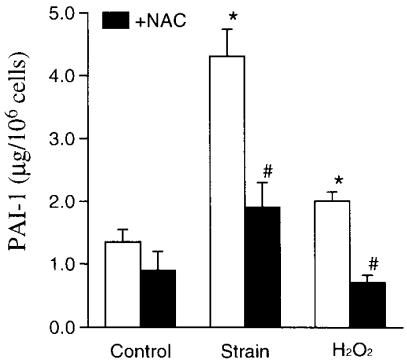

FIG. 3. Antioxidant inhibits the strain-induced PAI-1 release. ECs were pretreated with antioxidant (NAC, 20 mM) 0.5 h prior to strain treatment. After strain (12%) for 24 h, the culture media were collected and the concentrations analyzed by ELISA. ECs treated with H_2O_2 (100 μM) for 24 h were used as a reference for ECs under oxidant stress condition. Results are presented as mean±SEM from 3 to 5 experiments. *$P<.05$ vs unstrained control cells. #$P<.05$ vs respective strained or H_2O_2-treated cells.

intracellular catalase activity in strained cells, cell lysates were preincubated with 3-ATA (3-amino-1, 2, 4-triazole), a specific catalase inhibitor, prior to the analysis of H_2O_2 decomposition. As shown in Fig 2B, H_2O_2 decomposition activity in cell lysates pretreated with 3-ATA was almost entirely inhibited. These results indicate that strain can specifically induce intracellular ROS generation with a concomitant increase in catalase activity. To demonstrate that ROS is involved in the strain-induced PAI-1 secretion, ECs were pre-treated with NAC, prior to strain treatment. As shown in Fig 3, NAC treatment in control unstrained groups did not cause a significant decrease in PAI-1 secretion. In contrast, the strain-induced PAI-1 secretion from strained cells was greatly inhibited. To further confirm that ROS was operative in the strain-induced PAI-1 secretion, ECs were treated with H_2O_2 (100 μM) for 24 h. As indicated, H_2O_2-treated ECs increased its PAI-1 secretion. This H_2O_2-induced PAI-1 secretion could also be inhibited by pre-treating ECs with NAC. Taken together, these results indicate that cyclic strain applied to ECs can specifically induce ROS generation and the increased ROS levels may in turn be involved in the strain-induced PAI-1 secretion.

DISCUSSION

PAI-1 serves as the major physiologic inhibitor of plasminogen activator, and attenuates the fibrinolytic activity of the plasminogen activation (23). PAI-1 secretion from ECs is under the control of numerous growth factors, cytokines and hormones (24). Because ECs are constantly subjected to the influence of blood flow dynamics, many effects of hemodynamic force on fibrinolytic activities of ECs have been reported (1-4). In contrast to the shear stress effects, the secretion of PAI-1 can be stimulated by cyclic strain treatment (3, 4). The molecular mechanism that leads to the discrepancy in their secretion remains unclear. Recent studies indicate that higher concentration of PAI-1 together with lower levels of tPA, are characteristic of advanced atheromatous lesions (25, 26). Elevated PAI-1 levels in patients with essential and pulmonary hypertension (22, 27, 28) have also been reported. The mechanism(s) that results in the elevated PAI-1 levels has not been clearified.

Recent evidence suggests that ROS may act as second messengers in endothelial cells for expression of various proteins including MCP-1 (10, 17) and ICAM-1 (17, 29). Although the actual mechanisms are not clear, ROS activation of the NF-κB and AP-1 transcription factors has been reported (10, 11, 12, 29). Oxidizied low density lipoprotein as well as tumor necrosis factor can induce oxidant stress in ECs. Both are known to stimulate the PAI-1 secretion (30,

31). The mechanism whereby the cells sense the mechanical strain and stimulate PAI-1 release remain unclear. However, an upstream regulatory region of the PAI-1 gene contains a functional AP-1 binding site (32). It is possible that cyclic strain-induced ROS regulates PAI-1 release by inducing gene expression through activation at this AP-1 binding site. However, the ROS merely exerting its effect on secretory process can't be excluded. For the first time our data indicate that mechanical forces can induce ROS generation. This induced ROS generation appears to be strain-dependent since it was maintained at an elevated level as long as the strain remained and returned to basal levels with the removal of strain. ECs under strain for 24 h remained intact as revealed from morphology and non-significant increase in lactate dehydrogenase release. Due to the damaging potential of ROS, ECs depend on antioxidant mechanisms to rapidly metabolize these toxic intermediates and to prevent significant free radical injury. Among these defense mechanisms, catalase, was rapidly activated to remove strain-induced H_2O_2. This induced catalase activity is also strain-dependent since it returned to basal levels after the decline of ROS levels.

Atherosclerotic lesions are preferentially localized at bifurcated and curved regions of the arterial tree. Recent analysis of stress concentration and strains in the arterial walls have indicated that strains are significantly higher at branch sites than in the straight segments (33). The strain-induced PAI-1 release and its anti-fibrinolytic activity may predispose these branched regions to the development of atherosclerotic plaque. Evidence suggests that hypertension accelerates atherosclerosis in part because of synergy between elevated blood pressure and other atherogenic stimuli to induce oxidative stress on the arterial wall (34). Involvement of ROS in the development of hypertension has been implicated by the finding that superoxide dismutase targeting vessel walls reduces the blood pressure in spontaneously hypertensive rats (35). Our results of ROS generation by strain and the strain-induced PAI-1 release strongly suggest that oxidant stress may play an important role in hemodynamic force-induced pathological states including atherosclerosis, hypertension and reperfusion-induced vessel damage. The rapid response of ROS is also likely to be a key factor which regulates the acute responses and/or gene expression in ECs under mechanical deformation during cardiovascular interventions such as balloon angioplasty. The inhibitory effect of antioxidant on the PAI-1 release may also provide a potential mechanism for the cardioprotective effects of the antioxidant in various cardiovascular disorders.

In summary, our study demonstrates that intracellular ROS level can be induced by cyclic strain and this ROS generation is involved in the strain-induced PAI-1 release. These results also indicate the potential importance of intracellular ROS in the hemodynamic forces-induced cellular response in vascular ECs. The mechanism by which cyclic strain leads to increased ROS and subsequent modulation of endothelial fibrinolytic activities remains an important question which warrents further study.

REFERENCES

1. Davies, P. F. (1995) *Physiological Rev.* **75**(3), 519–560.
2. Frangos, J. A., Ed. (1993) Physical Forces and the Mammalian Cell, Academic Press, San Diego.
3. Diamond, S., Eskin, S. G., and McIntire, L. V. (1989) *Science* **243,** 1483–1485.
4. Carosi, J. A., McIntire, L. V., and Eskin, S. G. (1994) *Biotechnology and Bioengineering* **43,** 615–621.
5. Wang, D. L., Tang, C. C., Wung, B. S., Chen, H. H., Hung, M. S., and Wang, J. J. (1993) *Biochem. Biophys. Res. Commun.* **195,** 1050–1056.
6. Wang, D. L., Wung, B. S., Peng, Y. C., and Wang, J. J. (1995) *J. Cell. Physiol.* **163,** 400–406.
7. Wang, D. L., Wung, B. S., Shyy, Y. J., Lin, C. F., Chao, Y. J., Usami, S., and Chien, S. (1995) *Circu. Res.* **77,** 294–302.
8. Wung, B. S., Cheng, J. J., Chao, Y. J., Lin, J., Shyy, Y. J., and Wang, D. L. (1996) *Am. J. Physiol.* **39**(270), H1462–H1468.
9. Cheng, J. J., Wung, B. S., Chao, Y. J., and Wang, D. L. (1996) *Hypertension* (in press).

10. Satriano, J. A., Shuldiner, M., Hora, K., Xing, Y., Shan, Z., and Schlondorff, D. (1993) *J. Clin. Invest.* **92,** 1564–1571.
11. Schreck, R., Rieber, P., and Baeuerle, P. A. (1991) *The EMBO J.* **10**(8), 2247–2258.
12. Collins, T. (1993) *Lab Invest.* **68**(5), 499–508.
13. Li, Y., and Jaiswal, A. K. (1994) *Eur. J. Biochem.* **226,** 31–39.
14. Khachigian, L. M., Resnick, N., Gimbrone, M. A., and Collins, T. (1995) *J. Clin. Invest.* **96,** 1169–1175.
15. Sumpio, B. E., Du, W., and Wu, W. (1994) *Endothelium.* **2,** 149–156.
16. Laurindo, F. R. M., Pedro, M. A., Barbeiro, H. F., Pileggi, F., Carvalho, M. H. C., Augusto, O., and deLuz, P. L. (1994) *Circu. Res.* **74,** 700–709.
17. Wang, D. L., Cheng, J. J., Wung, B. S., and Chao, Y. J. (1996) *FASEB J.* **10**(3), A563.
18. Banes, A. J., Link, G. W., Gilbert, J. W., Tay, R. T. S., and Monbureau, O. (1990) *Am. Biotech. Lab.* **8,** 12–22.
19. Bass, D. A., Parce, J. W., Dechatelet, L. R., Szejda, P., Seeds, and Thomas, M. (1983) *J. Immunol.* **130**(4), 1910–1917.
20. Sundaresan, M., Yu, Z-Y., Ferrans, V. J., Irani, K., and Finkel, T. (1995) *Science* **270,** 296–299.
21. Aebi, H. (1984) *Methods Enzymol.* **105,** 121–126.
22. Wang, D. L., Pan, Y. T., Wang, J. J., Cheng, C. H., and Liu, C. Y. (1993) *Thromb and Haemostas.* **71**(4), 493–498.
23. Wiman, B. (1995) *Thromb. Haemost.* **74,** 71–76.
24. Johnson, M. R., Bruzdzinski, C. J., Winograd, S. S., and Gelehrter, T. D. (1992) *J. Biol. Chem.* **267**(17), 12202–12210.
25. Hamsten, A., Wiman, B., deFaire, U., and Bloomback, M. (1985) *N. Engl. J. Med.* **313,** 1557–1563.
26. Robbie, L. A., Booth, N. A., Brown, P. A. L., and Bennett, B. (1996) *Arteriocler. Thromb. Vasc. Biol.* **16,** 539–545.
27. Donders, S. H., Lustermans, F. A., and Van Wersch, J. W. (1992) *Blood Coagul. Fibrinolysis.* **3**(1), 61–67.
28. Eisenberg, P. R., Lucore, C., Kaufman, L., Sobel, B. E., Jaffe, A. S., and Rich, S. (1990) *Circulation.* **82**(3), 841–847.
29. Roebuck, K. A., Rahman, A., Lakshminarayanan, Janakidevi, K., and Malik, A. B. (1995) *J. Biol. Chem.* **270**(32), 18966–18974.
30. Latron, Y., Chautan, M., Anfosso, F., Alessi, M. C., Nalbone, G., Lafont, H., Juhan-Vague, I. (1991) *Artheioscler. Throm.* **11**(6), 1821–1829.
31. Van-Hinsbergh, V. W., Van-den-Berg, E. A., Fiers, W., and Dooijewaard, G. (1990) *Blood.* **75**(10), 1991–1998.
32. Descheemaeker, K. A., Wyns, S., Nelles, L., Auwerx, J., Ny, T., and Collen, D. (1992) *J. Biol. Chem.* **267**(21), 15086–15091.
33. Thubrikar, M. J., Roskelley, S. K., and Eppink, R. T. (1990) *J. Biochem.* **23,** 15–26.
34. Alexander, R. W. (1995) *Hypertension.* **25,** 155–161.
35. Grunfeld, S., Hamiltons, C. A., Mesaros, S., McClain, S. W., Dominiczak, A. F., Bohr, F. D., and Malinski, T. (1995) *Hypertension.* **26**(part 1), 854–857.