quence of the effects of the trauma of setting up the preparation, although this did not appear to affect ATP release. Immunohistochemical analysis of rat mesenteric resistance vessels has demonstrated the presence of SP-immunoreactive nerve fibres[33], however, it is unlikely that the source of the SP is from perivascular nerves. Indeed, in the perfused rat hindlimb preparation, destruction of SP-containing sensory nerves with capsaicin has no effect on the levels of SP released during increased flow[20].

In rat mesenteric arteries, it is possible that ATP has a more important role than SP as an endothelium-dependent relaxing agent since it has a potent relaxing action via endothelial P_{2Y}-purinoceptors[23], while SP is a poor dilator in this preparation[1]. A physiological role for ATP has also been proposed in the initiation of hypoxic vasodilatation of the coronary vasculature since specific blockade of P_{2Y} receptors attenuated dilatation due to ATP and to hypoxia, an action mediated via the release of EDRF[34].

This study, showing the release of ATP and SP into the perfusate of the rat mesenteric arterial bed, is in accordance with an increasing number of studies which show that endothelial cells are not only effectors, but a source of vasoactive substances. Furthermore, our study confirms that increased flow is an effective stimulus to evoke their release. This, coupled with the fact that an increase in flow causes an endothelium-dependent relaxation in many vessels, and with evidence that ATP and SP initiate endothelium-dependent relaxation in many vessels, makes it possible that substances released from endothelial cells may have a physiological role as mediators of flow-induced dilatation.

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1 Kawasaki, H., Takasaki, K., Saito, A., and Goto, K., Nature *335* (1988) 164.
2 Furchgott, R. F., and Zawadski, J. V., Nature *288* (1980) 373.
3 Furchgott, R. F., Circ. Res. *53* (1983) 557.
4 Smiesko, V., Kozik, J., and Dolezel, Š., Blood Vessels *22* (1985) 247.
5 Holtz, J., Forstermann, U., Pohl, U., Giesler, M., and Bassenge, E., J. cardiovasc. Pharmac. *6* (1984) 1161.
6 Rubanyi, G. M., Romero, J. C., and Vanhoutte, P. M., Am. J. Physiol. *250* (1986) H1145.
7 Kaiser, L., Hull, S. S., and Sparks, H. V. Jr, Am. J. Physiol. *250* (1986) H974.
8 Hull, S. S., Kaiser, L., Jaffe, M. D., and Sparks, H. V., Blood Vessels *23* (1986) 183.
9 Griffith, T. M., Edwards, D. H., Davies, R. L., Harrison, T. J., and Evans, K. T., Nature *329* (1988) 442.
10 Smiesko, V., Lang, D. J., and Johnson, P. C., Fedn Proc. *46* (1987) 643.
11 Peach, M. J., Loeb, A. L., Singer, H. A., and Saye, J., Hypertension, suppl. I, *7* (1985) I94.
12 Vanhoutte, P. M., Rubanyi, G. M., Miller, V. M., and Houston, D. S., Ann. Rev. Physiol. *48* (1986) 307.
13 Burnstock, G., Lincoln, J., Fehér, E., Hopwood, A. M., Kirkpatrick, K., Milner, P., and Ralevic, V., Experientia *44* (1988) 705.
14 Linnik, M. D., and Moskowitz, M. A., Peptides *10* (1989) 957.
15 Ralevic, V., Lincoln, J., and Burnstock, G., in: Endothelial Regulation of Vascular Tone. Eds U. S. Ryan and G. M. Rubanyi. Marcel Dekker, New York 1990.
16 Parnavelas, J. G., Kelly, W., and Burnstock, G., Nature *316* (1985) 724.
17 Loesch, A., and Burnstock, G., Anat. Embryol. (Berl.) *178* (1988) 137.
18 Milner, P., Ralevic, V., Hopwood, A. M., Fehér, E., Lincoln, J., Kirkpatrick, K. A., and Burnstock, G., Experientia *45* (1989) 121.
19 Lincoln, J., Loesch, A., and Burnstock, G., Cell Tissue Res. *259* (1990) 341.
20 Ralevic, V., Milner, P., Hudlická, O., Kristek, F., and Burnstock, G., Circ. Res. *66* (1990) 1178.
21 Milner, P., Kirkpatrick, K. A., Ralevic, V., Toothill, V., Pearson, J. D., and Burnstock, G., Proc. Roy. Soc. B *241* (1990) 245.
22 Milner, P., Bodin, P., Loesch, A., and Burnstock, G., Biochem. biophys. Res. Commun. *170* (1990) 649.
23 Ralevic, V., and Burnstock, G., Br. J. Pharmac. *95* (1988) 637.
24 Kirkpatrick, K., and Burnstock, G., Eur. J. Pharmac. *138* (1987) 207.
25 Burnstock, G., Pharmac. Rev. *24* (1972) 509.
26 Burnstock, G., Trends Pharmac. Sci. *9* (1988) 116.
27 Gordon, J. L., Biochem. J. *233* (1986) 309.
28 Rapaport, E., and Fontaine, J., Proc. natl Acad. Sci. USA *86* (1989) 1662.
29 Pearson, J. D., and Gordon, J. L., Nature *281* (1979) 384.
30 Rubanyi, G. M., Romero, J. C., and Vanhoutte, P. M., Am. J. Physiol. *250* (1986) H1145.
31 Bevan, J. A., and Joyce, E. H., Blood Vessels *25* (1988) 101.
32 Bodin, P., Bailey, D. J., and Burnstock, G., Br. J. Pharmac. *103* (1991) 1203.
33 Scott, T. M., Robinson, J., and Foote, J., J. Anat. *162* (1989) 177.
34 Hopwood, A. M., Lincoln, J., Kirkpatrick, K. A., and Burnstock, G., Eur. J. Pharmac. *165* (1989) 323.

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The endothelium-dependent relaxation of human middle cerebral artery: Effects of activated neutrophils

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Abstract. Neutrophils, activated by 4β-phorbol-12β-myristate-13α-acetate, decreased acetylcholine-induced relaxation of strips of human middle cerebral artery precontracted with noradrenaline. This effect was prevented by catalase, but not by superoxide dismutase. Nifedipine, propranolol and, less markedly, captopril reduced the decrease in acetylcholine-induced relaxation. Aspirin and dipyridamole did not reduce it.
Key words. Neutrophils; endothelium; oxygen products; regulation by drugs.

Vascular endothelium contains a humoral factor (or factors) which causes relaxation of vascular smooth muscle and mediates effects of many vasoactive substances[1]. Accordingly, damage to the vascular endothelium was considered a very important reason for circulatory diseases in atherosclerosis[2] and diabetes[3]. It is suggested that the functional damage to the endothelium may be brought about by activated neutrophils. Several studies have shown the ability of reactive oxygen products from activated neutrophils to damage cultured endothelial cells[4, 5]. The objective of the present study was to determine whether neutrophils are able to interfere with endothelium-dependent vascular relaxation of human cerebral arteries.

Materials and methods

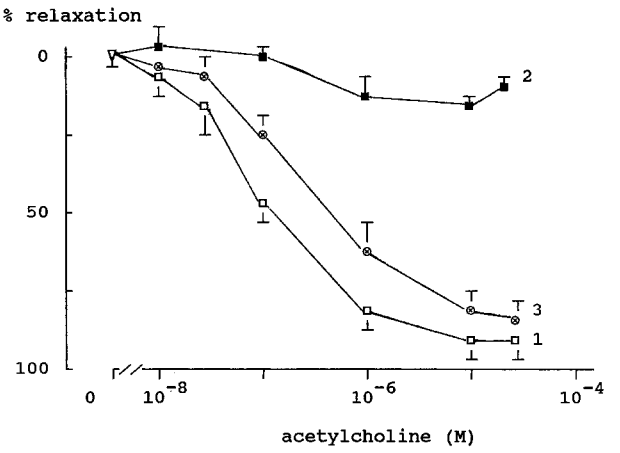
Human middle cerebral arteries (MCA) were removed by dissection within 2 h after death and were immersed in sterile Krebs-Ringer solution for immediate investigation. Vessels of adult subjects without primary cerebrovascular disease (the causes of death included drowning, generalized trauma, dissecting aortic aneurysm) were used. Helically cut strips of MCA (20 mm in length) were prepared and contractions were registered with an isotonic lever system. In some experiments the endothelium of the strips was removed by carefully scraping the intimal surface with a razor blade. The bath medium (tris buffered solution[6], composition (mM):NaCl 118.0, KCl 5.9, $CaCl_2$ 2.5, $MgSO_4$ 1.2, glucose 10.0, and tris 5.0, pH 7.4) was changed every 10 min throughout the experiment.

MCA strips were precontracted with noradrenaline (NA, 10^{-7} M). Acetylcholine (ACh, 10^{-8}–10^{-5} M) was added to induce endothelium-dependent relaxation[7]. Neutrophils without or with drugs were then added to the bath medium (final concentration 5×10^6 cells/ml) for 40 min (4 wash-out periods). After that, the bath medium was changed twice before the next contraction-relaxation period.

Human neutrophils were obtained from venous blood of healthy adult volunteers and separated by dextran sedimentation[8]. To activate neutrophils, 4β-phorbol-12β-myristate-13α-acetate (PMA, 10^{-7} M) was used.

Results

ACh induced concentration-dependent relaxation of the MCA strips precontracted with NA (fig.). In strips without endothelium, ACh had no effect. After adding non-activated neutrophils the ACh effects did not change. PMA alone had no effect on ACh-induced relaxation, either. However, after the treatment of MCA strips with neutrophils activated by PMA, the vasodilator effect of ACh was statistically reduced ($p < 0.01$, fig.). After removal of activated neutrophils, subsequent addition of ACh did not induce vascular relaxation, even at 3 h after the removal. If neutrophils were added to the bath fluid together with catalase (1850 units), the inhibition of the relaxation induced with ACh was less marked ($p < 0.05$, fig.). In contrast, the inclusion of superoxide dismutase (SOD, 280 units) in the bath fluid did not change the decrease in the ACh relaxation (not shown).



Effect of activated neutrophils on acetylcholine-induced relaxation of human middle cerebral artery strips precontracted by noradrenaline. (1) Control effect of acetylcholine. (2) Effect after the treatment of strips by activated neutrophils. (3) Effect after the treatment of strips by activated neutrophils in presence of catalase (1850 units).

In order to study drugs acting on neutrophil-induced disturbance of ACh relaxation of MCA strips, some drugs were added to the bath fluid simultaneously with activated neutrophils. It was found that the decrease of relaxation brought about by activated neutrophils was antagonized by nifedipine, propranolol, and captopril at high concentration only, but aspirin and dipyridamole did not significantly change the decrease (table).

Discussion

The present experiments show that activated neutrophils caused a disturbance in ACh-induced endothelium-de-

Effects of some drugs on ACh-induced relaxation of human middle cerebral artery after exposure to activated neutrophils.

Drug	The decrease in ACh-induced relaxation in percent	
	Exposure to neutrophils without drug	Exposure to neutrophils with drug
Nifedipine		
10^{-7} mol/l	81.4 ± 5.8 (16)	49.9 ± 3.7 (10)**
10^{-5} mol/l	78.4 ± 4.2 (17)	34.7 ± 2.3 (17)***
Propranolol		
10^{-7} mol/l	78.4 ± 5.9 (17)	53.2 ± 3.0 (11)**
10^{-5} mol/l	80.2 ± 6.1 (15)	39.6 ± 4.3 (14)***
Dipyridamole		
10^{-7} mol/l	78.4 ± 5.9 (13)	75.8 ± 6.2 (7)
10^{-5} mol/l	79.9 ± 5.1 (17)	60.0 ± 7.0 (10)
Captopril		
10^{-7} mol/l	77.2 ± 4.7 (14)	69.4 ± 6.7 (9)
10^{-5} mol/l	79.5 ± 5.2 (20)	53.6 ± 4.8 (14)*
Aspirin		
10^{-5} mol/l	78.7 ± 4.9 (20)	72.4 ± 6.9 (14)

* $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ versus neutrophil effects without drug. In brackets: number of experiments. Concentration of ACh–10^{-7} mol/l.

pendent vasorelaxation. This effect appears to depend on structural damage to the endothelium, because the removal of neutrophils does not restore the ability of ACh to induce vasorelaxation. Our results confirm the data of Varani et al.[5] that H_2O_2 plays the main role in this process, because catalase but not SOD protected the endothelium from damage. It is likely that in general the damage to the endothelium is induced by HO^- generated from H_2O_2[5].

Our results revealed that neutrophils can damage the endothelium to a sufficient extent to provoke a disturbance of vascular reactivity, in particular a decrease in ACh-induced vasorelaxation. Accordingly, pharmacological treatment to counter neutrophil actions on vascular endothelium might be considered as a method for the prevention of vascular disease. The present study shows that calcium channel blockade using nifedipine, and beta-adrenoreceptor blockers like propranolol may be able to reduce the deleterious effects of activated neutrophils on endothelium-dependent vasorelaxation.

In conclusion, while it may be extremely difficult to obtain definitive in vivo evidence for the involvement of neutrophils in processes leading to a disturbance of endothelium-dependent vasorelaxation, our findings are consistent with the suggestion that intravascular activation of neutrophils leads to tissue damage, including direct cytotoxic effects on endothelial cells[4, 5, 9].

1 Vane, J. R., Gryglewski, R. J., and Bunting, R. M., TIPS *8* (1987) 491.
2 Sreeharan, N., Jayakody, R., and Senarathe, M., Can. J. Physiol. Pharmac. *64* (1986) 1451.
3 Oyama, Y., Kawasaki, H., Hattori, Y., and Kanno, M., Eur. J. Pharmacol. *132* (1986) 75.
4 Weiss, S. J., Young, J., LoBuglio, A. F., Slivka, A., and Nimeh, N. F., J. clin. Invest. *68* (1981) 714.
5 Varani, J., Fligiel, S., Till, G. O., Kunkel, R. G., Ryan, U. S., and Ward, P. A., Lab. Invest. *53* (1985) 656.
6 Karaki, H., and Weiss, G., Arch. int. Pharmacodyn. *252* (1981) 29.
7 Forstermann, U., and Neufang, B., Br. J. Pharmac. *82* (1984) 765.
8 Walsh, C. E., Waite, B. M., Thomas, M. J., and Chatelet, L. R., J. biol. Chem. *256* (1981) 7228.
9 Ward, P. A., Till, G. O., Kunkei, R., and Beauchamp, C., J. clin. Invest. *72* (1983) 789.

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Changes in erythrocyte membrane lipid composition affect the transient decrease in membrane order which accompanies insulin receptor down-regulation

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Abstract. We have recently demonstrated, using electron paramagnetic resonance (EPR) spectroscopy, that insulin receptor internalization in response to insulin incubation (down-regulation) in human erythrocytes is accompanied by a transient decrease in membrane order, as measured by the $2T'_{||}$ order parameter. Since membrane lipids play such an important role in receptor internalization, we investigated the possible effects that an alteration of the normally-occurring lipid profile might have on down-regulation and the concomitant transient decrease in membrane order. Consequently, human erythrocytes enriched with cholesterol and erythrocytes from cirrhotic patients were examined, because both of these groups of cells have a higher cholesterol/phospholipid molar ratio (CH/PL) than controls. The 5-nitroxystearate spin label, which inserts into the lipid bilayer of cell membranes, was used to monitor changes in $2T'_{||}$ for a 3-h period at 37 °C. We report here that both cholesterol-enriched and cirrhotic erythrocytes do not down-regulate, as demonstrated by binding assays, and that they do not show the typical transient decrease in membrane order observed in controls. The results seem to indicate that a more ordered membrane inhibits internalization of the insulin receptor in erythrocytes, and that an increase in membrane disorder is necessary for insulin receptor down-regulation.
Key words. Erythrocyte; insulin receptor; receptor endocytosis; EPR; membrane order; membrane lipids.

We have recently demonstrated by electron paramagnetic resonance (EPR) spectroscopy, using 5-nitroxystearate as spin label, that incubation of human erythrocytes with insulin (1 μg/ml) results in a marked decrease (within the first 90 min) of membrane order as measured by the $2T'_{||}$ order parameter[1]. This parameter, which then returns to its initial value in less than 3 h, completes a cycle that appears to be related to the endocytotic internalization of