Transcytosis inhibitor N-ethylmaleimide increases microvascular permeability in rat muscle

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Carlsson, Ola, Bert-Inge Rosengren, and Bengt Rippe. Transcytosis inhibitor N-ethylmaleimide increases microvascular permeability in rat muscle. Am J Physiol Heart Circ Physiol 281: H1728–H1733, 2001.—N-ethylmaleimide (NEM) has been claimed to markedly inhibit the transvascular passage of small proteins and albumin by interacting with the docking and fusion of plasmalemmal vesicles with their target membranes. To investigate the role of transcytosis in the transcapillary passage of albumin, we assessed the effects of NEM on 125I-labeled radiiodinated serum albumin clearance (RISA-Cl) from blood to muscle in isolated and maximally vasodilated perfused rat hindquarters, in which vascular pressures, pre- and postcapillary resistances, and the capillary filtration coefficient (CFC) were continuously monitored. NEM (0.3–0.5 mM) caused a marked increase mainly in precapillary vascular resistance. Thus the arterial-to-venous resistance ratio in NEM-treated animals was 3.12 ± 0.56 versus 1.66 ± 0.17 during the control period (P < 0.05). Despite that, there was a doubling of both CFC from 0.0363 ± 0.0028 to 0.0778 ± 0.0101 ml/min/mmHg, 100 g−1 (P < 0.01) and RISA-Cl, compared with the control situation, signaling markedly increased microvascular permeability. Our results strongly suggest that NEM, besides producing marked vasoconstriction, also causes damage to the capillary endothelium. Thus, instead of inhibiting transvascular transport, NEM may induce increases in the bulk transport of albumin from blood to tissue.

expected from the temperature-dependent increases in perfusate viscosity induced by the cooling procedure (23, 25). Also, perfusion fixation of the vasculature in isolated rat hindquarters did not alter the passage of albumin differently from the transport of small solutes and water, indicating that passive transport mechanisms be responsible for both small and large transcapillary solute transport (6). However, some specific substances, e.g., low-density lipoproteins, may be transported by transcytosis (25). In the latter case, transport is markedly altered by cooling action in single perfused frog capillaries.

During the last few years there have been some attempts to evaluate the role of transcytosis in transcapillary exchange by employing chemicals that are supposed to block vesicular transport (17–19, 26, 28). Such substances are usually highly toxic because they interact with vital cell functions. N-ethylmaleimide (NEM) is an alkylating agent that interferes with free sulfhydryl groups in cysteine residues, thus inhibiting the docking and fusion of plasmalemmal vesicles with their target membranes (10, 11). Indeed, NEM has been claimed to markedly inhibit the transvascular transport of small proteins and even small solutes and albumin in situ perfusion experiments. Logically, these results have been interpreted to support the transcytosis hypothesis (19, 26).

However, there have also been several studies that have shown other, more unspecific, effects of treatment with NEM. For example, NEM treatment has been reported to induce conformational changes in human serum albumin (9) or to inhibit transport via the Na+-K+-2Cl− cotransporters in red blood cells (12). Another recent study (15) showed that NEM caused inhibition of the function of microtubule proteins, an effect that may have severe consequences for all living cells.

We have chosen well-characterized and continually weighed isolated perfused rat hindquarter preparation (21–23) to determine the actions of NEM because it allows control of vascular pressures and repeated assessments of pre- and postcapillary resistances, capillary pressure (Pc), and capillary filtration coefficient (CFC) during the course of the experiment. It is also possible to obtain the blood to tissue clearance of ra-
diodinated serum albumin (RISA) in muscle samples. We have thus been able to evaluate the actions of NEM in a physiologically better characterized and controlled vascular bed than in previous in situ perfusion experiments (17, 19, 26), in which continuous and careful recordings of intravascular pressures and capillary filtration properties were not performed. Somewhat surprisingly, our data suggest that NEM, instead of reducing vascular permeability to albumin, causes marked increases in albumin clearance, conceivably due to toxic endothelial cell damage.

METHODS

Experiments were performed on 22 male Wistar rats (Mølle- legaard; Copenhagen, Denmark), with an average body weight of 281.7 ± 15.7 g (mean ± SD). The rats were allowed free access to food and water until the day of the experiment. Anesthesia was induced by an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt), and was maintained during the surgical procedure by repeated intravenous administration of the same drug (~15 mg/kg).

The rats were placed in supine position on a heating pad. The tail artery was cannulated for continuous arterial pressure (P_A) monitoring on a polygraph (Grass Instruments; Quincy, MA), and for administration of drugs during the preparation. The hindquarter preparation has been described in detail (22). The peritoneal cavity was opened by a midline abdominal incision and an evisceration was performed. The abdominal aorta and the inferior caval vein were freed from surrounding tissues from the level of the renal to the iliolumbar vessels. The hindquarter was isolated by mass ligatures just proximal to these latter vessels and the spinal channel and bone marrow were plugged with a cotton ball soaked in silicone. The tail and paws were excluded by tight ligatures so that the preparation consisted mainly of skeletal muscle. The abdominal aorta was cannulated and connected to an artificial perfusion system driven by a perfusion pump (Alitea-XV; Stockholm, Sweden). A T tube was inserted into the caval vein for venous outflow and continuous registration of venous pressure (P_V) on the polygraph. The hindquarter was then separated from the cranial half of the body and placed on a counterbalanced plate, and connected to a force displacement transducer (model FT03, Grass Instruments) for continuous weight recordings. An infusion pump (Harvard Apparatus; Natick, MA) was connected to the perfusion system for continuous infusion of NEM during the test period. The free end of the venous outflow system could be adjusted to set the venous outflow pressure at any desired level. The perfusate was a modified albumin-Tyrode perfusate, to which horse serum (SVA; Stockholm, Sweden) was added (100 ml/l). The perfusate was constantly bubbled with a mixture of 95% O_2-5% CO_2 and kept at 38°C. The Tyrode perfusate was composed of (in mM) 148 Na, 4.3 K, 0.83 Mg, 2.5 Ca, 133 Cl, 25 HCO_3, 0.5 H_2PO_4, 5.6 glucose, and 0.088 papaverine.

Perfusion flow was kept constant at 8.5–9.0 ml/min (per 100 g of tissue), except when the capillary filtration coefficient (CFC) or capillary pre- and postcapillary resistances were assessed. During the control period, i.e., the first 40 min of the experiment, at least four CFCs and one pre-to-postvascular resistance ratio (R_A/R_V) were obtained. In the control group (n = 6), phosphate-buffered saline (0.4 ml/min) was sham-infused to the perfuse with the use of an infusion pump (Harvard Apparatus) during the test period. In the test hindquarters, NEM was infused to obtain a final perfusate concentration between 0.31 and 0.5 mM. These concentrations were chosen because they were able to produce alterations in albumin clearance, whereas lower doses (0.1–0.2 mM) were without effect. For 0.25-mM NEM, the total vascular resistance remained unchanged between the sham period and the treatment period (2.34 ± 0.34 vs. 2.96 ± 0.50 ml·min⁻¹·mmHg·100 g⁻¹, n = 3) as the CFC and albumin clearance (gastrocnemius), which also remained unaltered during NEM treatment (0.0524 ± 0.0140 vs. 0.0517 ± 0.0101 mmHg·min⁻¹·100 g⁻¹ during NEM, n = 3, and 48.4 ± 3.13 vs. 59.5 ± 5.14 μl/min during NEM, respectively). The test doses were reduced to a minimum to minimize NEM-induced damage to the vasculature. Because there were no significant differences in results for concentrations between 0.31 and 0.5 mM, the data for 0.31, 0.37, and 0.5 mM were pooled (n = 10).

To avoid extensive damage to the vascular bed, the NEM infusion was stopped when P_A was more than doubled. This occurred after ~19 min (~5–24 min maximum). After 10 min of NEM infusion, or earlier in those animals where the NEM infusion was stopped due to marked increases in P_V, the perfusate was changed to one containing RISA (1 MBq/ml 125I-labeled human serum albumin, Isopharma; Kjeller, Norway). After 40 min of RISA perfusion, the hindquarter was rinsed for 5 min with the use of standard perfusate to rid the vascular compartment completely from RISA. The amount of RISA in the washout fluid was <1% of the original concentration in all experiments. A few experiments were also performed to measure the amount of tracer passing to the tissues after 5 min of RISA perfusion and 5 min of washout. Also, in these experiments the tracer concentration in the washout fluid was low (<2% of initial concentration). There was no evidence of any significant binding of albumin to vascular walls from the 5-min experiments, because the 5-min albumin clearance values did not significantly differ from those measured during 40 min.

CFC was assessed from the weight gain slope ensuing after 2–3 min of concomitant increases of both P_A and P_V. RISA clearance (gastrocnemius), which also remained unaltered during NEM treatment (0.0524 ± 0.0140 vs. 0.0517 ± 0.0101 mmHg·min⁻¹·100 g⁻¹ during NEM, n = 3, and 48.4 ± 3.13 vs. 59.5 ± 5.14 μl/min during NEM, respectively). The test doses were reduced to a minimum to minimize NEM-induced damage to the vasculature. Because there were no significant differences in results for concentrations between 0.31 and 0.5 mM, the data for 0.31, 0.37, and 0.5 mM were pooled (n = 10).

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All data were expressed as means ± SE. Statistics were obtained using one-way analysis of variance and post hoc testing using the Bonferroni technique where applicable or Student’s t-test (paired or unpaired comparisons). All statis-
tical calculations were made with the use of the computer software SPSS for Macintosh, version 6.1.1 (SPSS; Chicago, IL).

RESULTS

Vascular resistance (peripheral resistance units) was more than twofold higher during NEM treatment than during the sham (phosphate-buffered saline) infusion period in controls (8.49 ± 0.68, n = 9, vs. 2.87 ± 0.38 mmHg·min·100 g−1·ml−1, n = 6, respectively; P < 0.0001), whereas during the initial control period, there was no difference between the two groups (2.56 ± 0.27, n = 9, vs. 2.29 ± 0.37 mmHg·min·100 g−1·ml−1, n = 6, respectively; NS, not significant) (Fig. 1). The increase in vascular resistance was mainly on the pre-capillary side, because there was a doubling in $R_A/R_V$ in the NEM-treated animals from 1.66 ± 0.17 to 3.12 ± 0.56; n = 9 (P, 0.05) (Fig. 2), whereas the concomitant increase in capillary pressure was moderate (from 10.7 ± 0.88 to 16.5 ± 2.7 mmHg; n = 8, not significant) (Fig. 3).

During NEM treatment, there was a doubling of CFC compared with the situation in control (0.0778 ± 0.0101 ml·min⁻¹·mmHg⁻¹·100 g⁻¹, n = 10, during NEM treatment vs. 0.0363 ± 0.0028 ml·min⁻¹·mmHg⁻¹·100 g⁻¹, n = 6, in controls; P < 0.01). CFC was markedly increased within <10 min of NEM treatment in all experiments. However, before the treatment period, CFC was similar in the two groups (0.0410 ± 0.0033 ml·min⁻¹·mmHg⁻¹·100 g⁻¹, n = 6, in the control group, and 0.0355 ± 0.00295 ml·min⁻¹·mmHg⁻¹·100 g⁻¹, n = 10, in the NEM group) (Fig. 4).

Despite the vasoconstriction occurring during the test period, there was a highly significant increase in RISA clearance from blood to tissues in NEM-treated preparations compared with controls for all muscles tested (Fig. 5).

Because of the repeated CFC measurements during the control period, all preparations developed some edema (5–10 g/h). However, the degree of edema formation remained near control in all muscles exposed to NEM, except for the gracilis muscle (20.6 ± 2.7 ml/100 g, n = 6, vs. 28.1 ± 3.8 ml/100 g, n = 10; P < 0.05 in the NEM-treated group). For the other muscles, there was only a tendency toward increased edema formation in the NEM-treated animals, but there was no significant increase in edema formation (Fig. 6).

DISCUSSION

The major result of the present study, performed under strictly controlled experimental conditions, is that NEM (0.3–1.0 mM) during 5–10 min of exposure time, causes damage to the capillary endothelium as well as marked lumen reductions of mainly precapillary vessels. The present data thus seem to disagree with those from previous studies using NEM as a transcytosis inhibitor, where a major emphasis has been in assessing morphological instead of physiological parameters. Thus, in some of the earlier studies (16, 19), perfusion and NEM exposure were made in situ, under conditions where the distribution of organ

Fig. 1. Vascular resistance (PRU) for controls (n = 6) (open bars) and NEM-treated (n = 9) animals (solid bars) during the control and test period. ***P < 0.001, compared with control.

Fig. 2. Pre- and postcapillary resistance ratios during control and test period in controls (n = 6) (open bars) and N-ethylmaleimide (NEM)-treated (n = 9) animals (solid bars). *P < 0.05, compared with control.

Fig. 3. Capillary pressure from at least 3 different measurements in each hindquarter preparation during control and test period in controls (n = 4) (open bars) and NEM-treated (n = 8) animals (solid bars).
blood flow and vascular pressures were mostly unknown and uncontrolled.

Predescu and colleagues (16, 19) found a reduction of the content of labeled small proteins and albumin in tissue samples from murine heart after (5 + 5 min) local NEM infusion in situ (1 mM) compared with control. In our hindquarter experiments, NEM concentrations >0.5 mM could not be used because they caused marked edema formation, conceivably by inducing increases in vascular permeability. Although some earlier observations (16) indicate that NEM treatment for <5 min will maintain the vasculature intact, we observed an elevated CFC already during the first 5 min of NEM (0.5 mM) infusion in 5 of 10 NEM experiments performed. The present “threshold doses” of NEM caused effects on both vascular resistance and albumin clearance, whereas lower doses (<0.3 mM) were without effect.

In in situ perfused rat lungs, the apparent lung tissue uptake of albumin was reduced by as much as 40–50% after NEM infusion (1.5 min), whereas the apparent tissue uptake of inulin (molecular radius ~14 Å) was largely unchanged (26). Because NEM can induce constriction of resistance vessels (hence, constriction in the lungs) and can reduce the intravascular volume (20), it is possible that this is the reason for the decrease in apparent solute clearance that was reported in previous tissue uptake studies (17, 19, 26). For a macromolecule, the major portion of the apparent “tissue space” of the tracer initially during its tissue accumulation (in a tissue uptake study), is made up by the intravascular space. Efficient intravascular washout is therefore crucial in short-term experiments to rid the vasculature of tracer. A few minutes of washout after just a few minutes of tracer loading may not be enough. The reduction in tissue spaces of large solutes after NEM may have been caused either by a vasoconstriction-induced reduction in the intravascular space, such as in the lung, where vasoconstriction will not primarily induce derecruitment of microvessels (20), or by a vasoconstriction-induced derecruitment of capillaries (cf. muscle) (22). For small- and intermediate-size solutes (cf. inulin) the intravascular portion of total tissue space during tracer accumulation is usually negligible. Thus the apparent tissue space for inulin would not be significantly affected by NEM-induced reductions in intravascular volume. This may be the reason why the apparent inulin tissue space, in contrast to that of albumin, was not significantly affected by NEM in the in situ perfused rat lung, although vasoconstriction and reductions in intravascular volume may have occurred (20, 26). In the murine heart, however, the most prominent effect of (precapillary) vasoconstriction seems to be derecruitment of microvessels, and this may be the reason why Predescu and colleagues (16, 19) found that both intermediate size and large solute clearances were reduced by NEM.

In a study by Schnitzer et al. (26) it was, however, claimed that lung vascular resistance was largely un-
affected by NEM because during NEM infusion, the total perfusion pressure was largely unchanged. Still, NEM-induced vasoconstriction may be a reason for the decrease in apparent albumin flux in these studies. One important factor to take into consideration when utilizing the isolated perfused rat lung model is that the pressure drop along the lung vasculature proper is rather small (∼3–4 mmHg) for the perfusion flows normally employed (10 ml/g lung). This may represent less than one-third of the total pressure drop along the entire perfusion circuit (9–10 ml·min⁻¹·g⁻¹). For such a low total flow resistance, a rise in pulmonary vascular resistance by 25% will give an overall pressure increment of only 1 mmHg in the circuit, which is readily overlooked (24). Small perfusion pressure changes may thus be masked in the perfused lung model, unless PA and PV are continuously and very carefully monitored.

In this context, the arbitrary use of a short (3 min) washin and a short washout period in nonheparinized animals in Schnitzer’s studies implies that both vascular wall phenomena (possible tracer adsorption to the endothelium) and insufficient washout may be problems in their experiments. In the present experiments, the concentration of tracer during washout was followed carefully (down to below ~1% of initial concentration) to ensure proper rinsing of the vasculature. Furthermore, Schnitzer’s studies were performed without the presence of serum factors (orosomucoid) in the perfusate (7, 8), and the albumin clearance values presented were threefold higher in control than those found in serum-perfused lungs where orosomucoid was present (24). NEM applied for considerably longer periods of time than in the study of Schnitzer et al. (26). However, in a parallel study, when the isolated lung was perfused under highly controlled (constant flow) conditions, 0.13 mM of NEM given for 4 min actually caused marked increases in lung macromolecular permeability as well as in CFC (24). Thus, it seems that the lung microvasculature is, if anything, even more sensitive than that of skeletal muscle to any detrimental actions of NEM. Furthermore, in the isolated perfused lung there were no reductions in albumin transport either for very low doses of NEM (<0.1 mM) or for moderate doses of another transcytosis inhibitor, filipin (0.2–0.8 μg/ml) (24). These findings are essentially consistent with those in the present study. Below a certain threshold concentration of the transcytosis inhibitor, there were no reductions in albumin clearance. However, above this concentration, there were progressive increases in vascular permeability concomitant with signs of vasoconstriction.

How NEM affects vascular permeability is largely unknown. However, NEM is an alkylating agent that acts like a fixative, causing marked cross-linking of proteins, such as those of the cytoskeleton and in the plasmalemma. Thus, NEM can exert many unspecific actions in living cells, such as inhibition of cellular microtubule proteins and ATPases (1, 15). It is also possible that NEM binds directly to albumin and thereby alters its conformation and/or charge (9). Therefore, in the present study, to avoid interactions with the tracer albumin, NEM was added continuously via a constant infusion to the perfusion system immediately before the arterial cannula of the hindquarter preparation. The present study and a parallel one (24) clearly point out the unsuitability of using NEM as a transcytosis inhibitor at the whole organ level.
In conclusion, the present study strongly suggests that NEM, a substance commonly used for transcytosis inhibition, increases microvascular permeability in maximally vasodilated rat hindquarters in doses comparable to those employed in previous studies on muscle. NEM also causes vasoconstriction, preferentially in precapillary vessels, an action, that is most probably responsible for the reduced clearance of macromolecules previously observed under nonvasodilated conditions. However, at the endothelial level, NEM is likely to produce toxic cellular damage, and as a consequence, an increased passage of proteins from blood to tissue.

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REFERENCES