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Hypotonicity induces L-selectin shedding in human neutrophils

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Kaba, Nubia K., and Philip A. Knauf. Hypotonicity induces L-selectin shedding in human neutrophils. *Am J Physiol Cell Physiol* 281: C1403–C1407, 2001.— Expression levels of adhesion molecules on neutrophils are affected under various conditions, including ischemia, possibly because of associated increases in cell volume. We examined the effects of cell swelling in hypotonic media on the level of L-selectin (CD62L) and β_2-integrin (CD18) on human neutrophils. In hypotonic media, neutrophils shed L-selectin. The shedding was greatly reduced by 30 μM RO31-9790, the metalloprotease (sheddase) inhibitor. Hypotonicity-induced L-selectin shedding was also time and tonicity dependent. Decreasing tonicity caused increased shedding. In 0.6× medium (0.6× the normal tonicity of 300 mosmol/kgH_2O), shedding increased over a 2-h period, after which >70% of the neutrophils had lost L-selectin. In contrast to L-selectin, the level of β_2-integrin on the neutrophil surface was not significantly affected. Thus L-selectin shedding, which occurs on neutrophil activation and is usually accompanied by β_2-integrin upregulation, was selectively induced by hypotonicity without a corresponding effect on β_2-integrin.

hypertonic; tumor necrosis factor-α; CD62l; metalloprotease; cell swelling

THE SELECTIN AND INTEGRIN classes of adhesion molecules play essential roles, respectively, in the rolling of neutrophils and then firm adhesion to the endothelium (2, 25). Modulation of surface adhesion molecules, such as L-selectin and β_2-integrin, could alter the host defense response and the degree of inflammation, as well as the degree of injury caused by reperfusion after ischemia (9, 23). Neutrophils rapidly shed their L-selectin on stimulation with chemotactic agents (1, 25) and upregulate their β_2-integrin level (4). L-selectin shedding is mediated by proteolytic cleavage by a membrane-bound metalloprotease, "sheddase" (14, 15, 25). Rizoli et al. (15) have shown that cell shrinkage in hypertonic media causes L-selectin shedding in neutrophils by a mechanism that involves the sheddase as well as the p38 mitogen-activated protein kinase (MAPK). In contrast, very little is known about swelling-induced L-selectin shedding.

The role played by neutrophil swelling in ischemia-reperfusion is also not well understood. During ischemia, the fall in intracellular pH would be expected to activate Na^+/H^+ exchange (13), causing a net uptake of NaCl and water and thereby causing cell swelling. Chemotactic factors such as *N*-formyl-methionyl-leucyl-phenylalanine also stimulate the Na^+/H^+ exchanger in neutrophils (16, 21), leading to cell swelling (16). Swelling has been shown to occur in endothelial cells during hemorrhagic shock (12), and this swelling is inhibited by the Na^+/H^+ exchange inhibitor amiloride (13). Activation of Na^+/H^+ exchange during ischemia-reperfusion is also likely to take place in neutrophils, and should lead to swelling.

When the intracellular pH of neutrophils was lowered in vitro by exposing cells to media that were acidified by addition of HCl (24) or by increasing CO_2 (20), there was an increase in surface β_2-integrin (CD18), indicating neutrophil activation. If a similar increase occurs during ischemia-reperfusion, this could enhance neutrophil binding to endothelia and increase the inflammatory response. The same experiments showed an increase in forward light scatter as measured by flow cytometry, which was prevented by amiloride (24), indicating that activation of Na^+/H^+ exchange causes an increase in cell volume.

Because neutrophils play a significant role in ischemia-reperfusion injury (3, 7, 10), and because neutrophil swelling is likely to occur under these conditions, the aim of this study was to determine what effects, if any, cell swelling by itself, without the accompanying complications introduced by substantial changes in cell

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pH, has on the levels of L-selectin and β_2-integrin and how this might affect neutrophil behavior in the microcirculation.

MATERIALS AND METHODS

Materials. Neutrophil isolation medium (NIM) was obtained from Cardinal Associates (Santa Fe, NM). FITC-conjugated monoclonal anti-L-selectin (CD62L), anti-β_2-integrin (CD18), and isotype (mouse IgG1) antibodies were purchased from Beckman/Coulter (Miami, FL). RO31-9790 was a kind gift from Roche Products, Hertfordshire, UK. All other chemicals, including salts, were obtained from Sigma (St. Louis, MO) and were of the highest purity available.

Neutrophil isolation. Blood samples were obtained, with informed consent, from healthy donors by venipuncture with heparin as anticoagulant. Whole blood and NIM were brought to room temperature before use. Three milliliters of NIM reagent were placed in an 8-ml Falcon polystyrene round-bottom tube (Becton Dickinson, Franklin Lakes, NJ), and 3.5 ml of whole blood were carefully layered on them with a serological pipette. Tubes containing NIM and blood were centrifuged at room temperature for 40–50 min at 1,500 rpm (470 *g*) in a Sorvall RC5C Plus centrifuge (Kendro Laboratory Products, Newtown, CT). After centrifugation, there were two visible leukocyte bands in the NIM, the upper one being mononuclear cells and the lower one neutrophils. The upper NIM fraction and the mononuclear cells were aspirated and discarded. The neutrophil band was collected with a Pasteur pipette and suspended in a total volume of 5.0 ml in a calcium-free neutrophil isolation buffer (NIB; in mM: 135 NaCl, 4.2 KCl, 5.0 glucose, and 10.0 HEPES) containing 0.1% BSA (fraction V, γ-globulin free and low endotoxin). Osmolality of the buffer was adjusted to 290–310 mosmol/kgH_2O (measured with a Wescor vapor pressure osmometer) by using NaCl, and the pH was adjusted to 7.4 with NaOH. Neutrophils were washed three times in 5.0 ml NIB by centrifuging at room temperature for 10 min at 1,000 rpm (210 *g*). Before the last wash, 0.5 ml of the neutrophil suspension was removed and put in 4.5 ml of a 1:6 dilution of PBS with distilled water to hypotonically lyse erythrocytes. After 30 s, 1.5 ml of 4× PBS were added to the lysing suspension to restore isotonicity, and neutrophils were then counted with a hemocytometer. The rest of the neutrophil suspension, which was used for the experiments, did not undergo hypotonic lysis to remove erythrocytes, since they can be clearly differentiated with the flow cytometer.

L-selectin (CD62L) and β_2-integrin (CD18) measurement. Surface L-selectin was determined by flow cytometer. Cells (5×10^5) were incubated in calcium (1.1 mM $CaCl_2$) and magnesium (0.9 mM $MgCl_2$) containing NIB with decreasing tonicity (1.0×, 0.9×, 0.7×, 0.6×, 0.5×, 0.3×) for 2 h or in 0.6× medium for various time intervals. Control cells were incubated in 1.0× buffer for the same time intervals. Standard isotonic medium (1.0×) had an osmolality of 300 mosmol/kgH_2O. The hypotonic buffers were made by diluting the isotonic (1.0×) buffer with distilled, deionized and autoclaved water. The RO31-9790 cells were preincubated at 37°C for 30 min with 30 μM inhibitor (made from a 30 mM stock solution in DMSO) before being subjected to the 0.6× solution containing 30 μM RO31-9790 for 2 h. Tumor necrosis factor-α (TNF-α; 3 ng/ml)-treated cells were incubated at 37°C for 2 h. All samples were placed on ice after the incubation. For surface β_2-integrin measurements, neutrophils were incubated in 0.6× buffer or hypertonic (500 mosmol/kgH_2O) buffer (by adding 100 mM NaCl to the isotonic buffer) at 37°C for 2 h.

All neutrophil samples were washed once (by centrifugation and resuspension) with ice-cold, 1.0×, pH 7.4 buffer before labeling with the monoclonal antibodies. Then 5×10^5 neutrophils in 250 μl of medium were incubated with saturating amounts (20 μl) of FITC-conjugated anti-L-selectin or anti-CD18 antibodies for L-selectin or CD18 measurement, respectively. Cells were incubated for 45 min at 4°C on a rotating platform. Cells were then washed twice with ice-cold, 1.0× buffer at 4°C. A third wash was done with BSA-free 1.0× buffer, and cells were resuspended and fixed with 1.6% paraformaldehyde in this buffer. Cells were washed once with BSA-free buffer, resuspended at 10^6 cells/ml, and analyzed on an Epics Elite ESP flow cytometer (Coulter) using 488-nm excitation. Data analysis was done with an Epics Elite flow cytometry workstation version 4.5. Typically, 10^4 cells were analyzed per sample. Gating to exclude red blood cells was done in the dot plot window by virtue of the greater forward and side scatter of the neutrophils. For β_2-integrin, results were expressed as the mean fluorescence intensity of the cells counted (in arbitrary units). For L-selectin, the number of positive cells was determined by comparison of histograms with those obtained using FITC-conjugated isotype antibody as described by Preece et al. (14). In brief, a gate was placed in such a way that at least 99% of isotype control antibody-labeled cells fell below it.

Statistical analysis. Data are presented as means ± SE for *n* experiments as indicated. Significance was assessed using Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of hypotonicity on L-selectin shedding. With increasing incubation time in 1.0× (isotonic) medium at 37°C, the percentage of neutrophils positive for L-selectin decreased slightly (Fig. 1), falling from 95 ± 1% to 83 ± 3% after 2 h (Fig. 1), probably because of nonspecific activation due to the artificial incubation

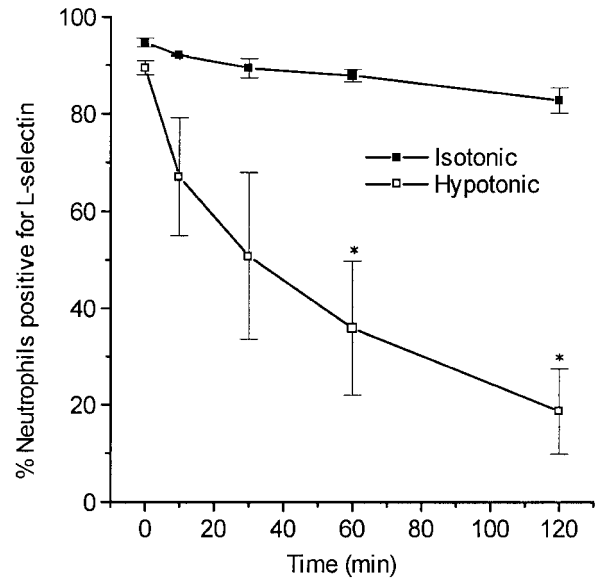

Fig. 1. Time course of L-selectin shedding in human neutrophils. Neutrophils were incubated at 37°C in 1.0× (isotonic) or 0.6× (hypotonic) medium for different times as described in MATERIALS AND METHODS. At *time 0*, samples were immediately placed on ice. Data are means ± SE of at least 3 separate experiments for each point. *$P < 0.05$ vs. isotonic.

conditions. In 0.6× medium (Fig. 1), the number of cells positive for L-selectin dropped from 90 ± 1% to 19 ± 9% within 2 h. The shedding was clearly detectable in 10–30 min and statistically significant after the first hour, during which over half of the neutrophils lost their L-selectin (Fig. 1).

The effect of incubating neutrophils for 2 h in media with various osmolarities is shown in Fig. 2. Neutrophils positive for L-selectin decreased from 80 ± 4% in 1.0× medium to 1 ± 0.2% in 0.3× medium. Shedding of L-selectin was significant in 0.7× medium and increased most sharply between tonicities of 0.9× and 0.6×.

In the presence of the sheddase inhibitor (RO31-9790), 74 ± 3% of neutrophils were positive for L-selectin after 2 h in 0.6× medium compared with only 29 ± 5% in 0.6× medium alone (Fig. 3). The inhibitor had no effect on the L-selectin level in isotonic medium. The 0.6× medium that was made isotonic by adding hypertonic NaCl solution had no effect on the baseline L-selectin level (not shown). This control rules out the possibility of nonspecific activation due to dilution of any of the buffer components or due to contaminants in the water that was added to make the medium hypotonic. As expected, because the sheddase is membrane bound, supernatant (made isotonic) from hypotonically treated cells also had no effect (not shown). Figure 3 also shows that 2 h incubation with 3 ng/ml TNF-α, a proinflammatory cytokine, caused the number of neutrophils positive for L-selectin to drop from 86 ± 2% to 1.3 ± 0.2%, a decrease similar to that caused by 0.3× medium, but lower than the value of 29 ± 5% seen with 0.6× medium (Fig. 3). Hypertonicity (500 mosM) for 2 h (15) had a similar effect on L-selectin as did 0.6× medium. For both hypotonicity (Fig. 1) and hypertonicity (15), the decrease was much slower than in the case of cytokines, where a near-maximum effect is usually seen within 30 min (1).

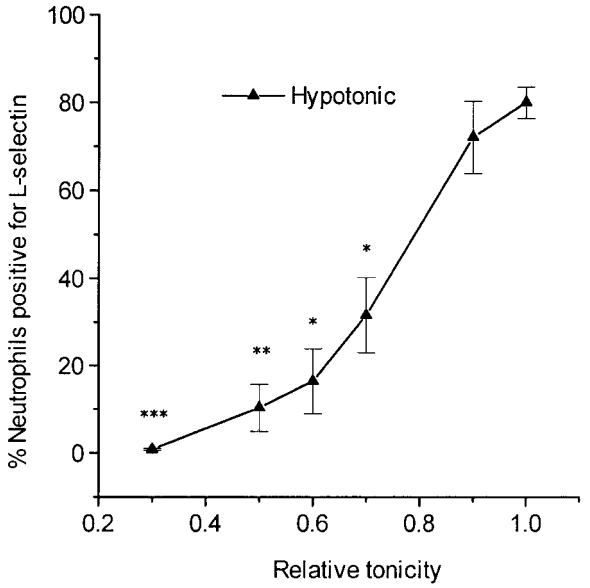

Fig. 2. Effect of decreasing tonicity on L-selectin shedding in neutrophils. Neutrophils were incubated at 37°C for 2 h in various tonicity media as described in MATERIALS AND METHODS. Data are means ± SE of at least 3 separate experiments for each point. *$P <$ 0.05, **$P < 0.001$, ***$P < 0.0001$ vs. isotonic.

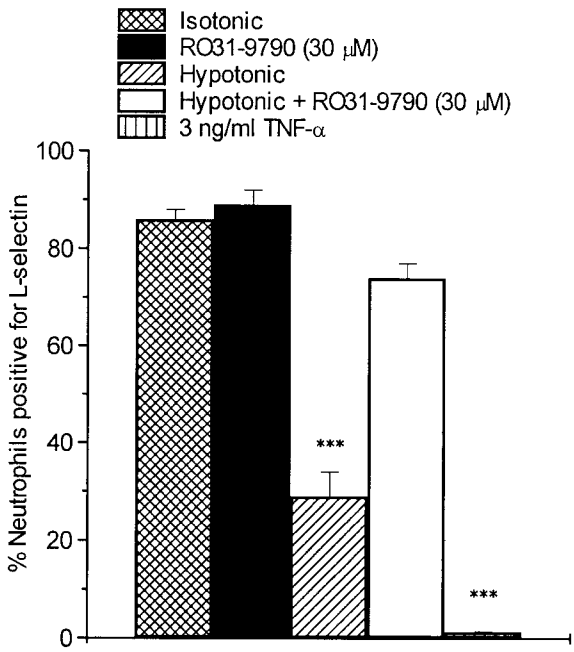

Fig. 3. Effect of hypotonicity, tumor necrosis factor-α (TNF-α), or RO31-9790 on L-selectin shedding in neutrophils. Neutrophils were incubated at 37°C for 2 h in 3 ng/ml TNF-α or in 0.6× (hypotonic) medium with or without 30 μM of the sheddase inhibitor RO31-9790. Data are means ± SE of at least 3 separate experiments. ***$P <$ 0.0001 vs. isotonic.

Effect of hypotonicity and hypertonicity on β_2-integrin (CD18) level in neutrophils. Incubation of neutrophils for 2 h in 0.6× medium or in a hypertonic (by adding 100 mM NaCl to the isotonic solution) medium had no significant effect on the β_2-integrin level, but 3 ng/ml of TNF-α showed a significant increase in β_2-integrin (Fig. 4).

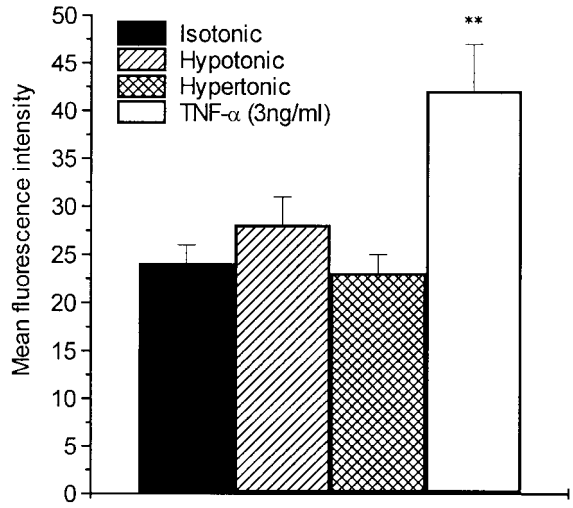

Fig. 4. Effect of hypertonicity (500 mosmol/kgH_2O), hypotonicity (0.6×), or TNF-α on neutrophil CD18 surface expression. Neutrophils were subjected to 0.6× medium, 3 ng/ml TNF-α, or hypertonic medium at 37°C for 2 h. Hypertonicity was achieved as described in MATERIALS AND METHODS. Data are means ± SE of at least 3 separate experiments. **$P < 0.001$ vs. isotonic.

DISCUSSION

Hypotonicity, which swells neutrophils (22), caused L-selectin shedding in a time- and osmolarity-dependent manner. The inhibition of the shedding by the metalloprotease (sheddase) inhibitor RO31-9790 shows that the sheddase is involved in the process. In addition, the fact that supernatant from hypotonically treated cells failed to shed L-selectin indicates that the effect does not involve any factors secreted from the cells, and is consistent with activation of the specific membrane-bound enzyme that mediates activator-induced shedding. Although most chemotactic agents downregulate L-selectin levels (25) and concomitantly upregulate β_2-integrin levels (4) on neutrophil activation, hypotonicity (Fig. 3), like hypertonicity (15), had a selective effect on L-selectin, and did not influence the β_2-integrin level (Fig. 4). This indicates that L-selectin shedding and β_2-integrin upregulation are separately controlled and that volume changes activate some but not all of the pathways stimulated by cytokines. Considered in light of evidence that low pH increases the β_2-integrin level (20, 24), the lack of an effect of cell swelling on β_2-integrin suggests that this β_2-integrin upregulation is primarily caused by the decrease in pH, with little or no contribution from the resulting increase in cell volume.

As previously shown by Rizoli et al. (15), cell shrinkage decreases L-selectin in neutrophils by activating the sheddase via a pathway involving the p38 MAPK. In some cells, cell shrinkage also activates protein kinase C (8) and triggers several proteins involved in the MAPK cascade that leads to Jun NH_2-terminal kinase (JNK) activation (11). Cell swelling has been shown to stimulate myeloperoxidase exocytosis in neutrophils (18) and, in other cells, to activate protein kinase C, encourage tyrosine phosphorylation, and stimulate the MAPK cascade as well as activate JNK (8, 17). Considering the common pathways involved in both swelling and shrinkage, it is conceivable that the shedding observed with hypertonicity (15) and hypotonicity could involve a common downstream cascade, which then leads to the activation of the sheddase. The possible involvement of tyrosine phosphorylation in this process requires further investigation.

Ischemia, which is a consequence of hypoperfusion caused either by hemorrhagic shock or interruption of blood flow, leads to intracellular acidification that causes Na^+ influx through the Na^+/H^+ exchanger (12, 13). This event leads to endothelial cell swelling (12, 13), a process that is exacerbated during reperfusion (5). Under these conditions, a similar process could also cause neutrophil swelling and therefore a decrease in the L-selectin level. This would be expected to impair the ability of neutrophils to roll on venular endothelium, which should make it more difficult for activated neutrophils to adhere firmly to vessel walls under conditions of flow. The slow time course of the shedding induced by cell swelling alone (Fig. 1), however, indicates that, even for neutrophils within the ischemic region, the loss of L-selectin by this mechanism would require 1–2 h of ischemia. Perhaps more significantly, the swelling-induced shedding, because of its slow time course, could not decrease L-selectin sufficiently to have much effect on rolling of neutrophils entering a local ischemic region on reperfusion. Other events that take place simultaneously during ischemia and reperfusion, such as changes in pH and intracellular calcium, however, may speed up the process.

Our results demonstrate that L-selectin levels can be regulated separately from β_2-integrin expression, in agreement with studies of the effects of hypertonicity (15), metabolic inhibitors (6), and 5-nitro-2-(3-phenylpropylamino)benzoic acid (19). These findings provide an experimental means for selectively altering the surface density of L-selectin to test predictions about the effects this may have on the rolling behavior of neutrophils, without any additional complications caused by changing the level of β_2-integrins.

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