

Journal of Clinical and Basic Cardiology

An Independent International Scientific Journal



Journal of Clinical and Basic Cardiology 1998; 1 (1), 52-54

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Vascular endothelial growth factor (VEGF) and endothelin-3 (ET-3) activate cation currents in human umbilical vein endothelial cells

E. L. K. Smith, H. Lauder¹, T.-P. D. Fan¹, C. R. Hiley¹, A. H. Hainsworth

Proliferation of endothelial cells is an essential process in repair following vascular injury and also in angiogenesis and vasculogenesis. Factors that influence endothelial proliferation are thus of great clinical and pharmacological interest. The endogenous peptides vascular endothelial growth factor (VEGF) and endothelin-3 (ET-3, acting via ET_B receptor activation) both potently stimulate regrowth of human umbilical vein endothelial cells (HUVEC) following mechanical wounding *in vitro*. The aim of this study was to test for electrophysiological correlates of these trophic effects.

In single HUVEC, whole cell currents were activated within 10 min after exposure to either VEGF (20 pmol/L) or ET-3 (100 nmol/L) the reversal voltage being -59 mV in each case. Either VEGF or ET-3 induced currents in cells at the edge of a wound in more than half the cells studied. However, ET_B receptor agonist sarafotoxin S6c (40 nmol/L) did not. Thus the ET-3-induced currents are likely to be the result of ET_A receptor stimulation. ET-3 induced no current in cells distant from a regrowing boundary.

These observations are consistent with a single population of cation channels that are activated by either peptide and are weakly selective for potassium ions ($p_K/p_{Na} = 3$). The observed currents are probably not directly involved in the growth promoting effects of ET-3 and VEGF. *J Clin Bas Cardiol 1998; 1: 52-4.*

Keywords: endothelin, vascular permeability factor, electrophysiology.

Proliferation of the endothelial cells that line blood vessel walls is a vital repair process following any form of vascular damage, such as ischaemia-reperfusion, surgical angioplasty or venous grafting. It is also an important component of angiogenesis, the process of normal capillary out-growth. Endothelial proliferation is influenced by numerous chemical mediators [1] including the endogenous peptides vascular endothelial growth factor (VEGF) and endothelin-3 (ET-3), both of which have growth-promoting effects on vascular endothelial cells. The glycoprotein VEGF is a potent angiogenic factor, which also increases blood vessel permeability. Importantly, its mitogenic effects appear to be specific for endothelial cells (see [2]) and it is essential for normal embryonic vasculogenesis and angiogenesis [3, 4]. ET-3 (0.1–100 nmol/L) accelerates regrowth of human umbilical vein endothelial cells (HUVEC) in a wounding model of endothelial damage [5, 6, 7] with significant effects after just 2 hours of exposure to the peptide [7]. ET-1 (1–1000 nmol/L) on the other hand is without effect [5, 6, 7], except in the presence of an ET_A receptor antagonist [7], suggesting that acceleration of regrowth is mediated by an ET_B receptor. The aim of this study was to test for electrophysiological correlates of the trophic effects of VEGF and ET-3 in the wounded HUVEC model.

Methods

Confluent monolayers of HUVEC (pooled cells from Clonetics, TCS Biologicals, Buckingham, UK) were grown on 13 mm Thermanox™ coverslips in medium 199 at 37° C supplemented with 0.10–0.15 v/v fetal bovine serum (Gibco Life Technologies, Paisley, UK). Parallel strips of denudation were produced by scraping with a wounding “comb” has been described elsewhere [5–7], before coverslips were taken for either imaging or electrophysiological experiments. The effects

of VEGF on the regeneration of HUVEC following wounding of a previously confluent monolayer was quantified using a Seescan (Cambridge, UK) computerised image analysis system. Electrophysiological experiments were performed on HUVEC (passage number 2–6) in the period 1–8 h after wounding. Standard whole-cell recordings [9] were made at room temperature. The bath (extracellular solution) was slowly perfused with HEPES buffered saline containing (mmol/L): 135 NaCl, 2.5 KCl, 2.5 CaCl₂, 0.5 MgCl₂, 1.0 NaH₂PO₄, 10 glucose, 10 HEPES (pH 7.4). The pipette (intracellular solution) contained (mmol/L): 110 K gluconate, 20 KCl, 8.0 MgSO₄, 1.0 Na₄EGTA, 0.01 EDTA, 4.0 Na₂ATP, 0.5 Na₃GTP, 10 HEPES (pH 7.4). Stock solutions of peptides were made on the day of use and kept on ice, then freshly diluted just before use and applied in the bath solution for 10 min. ET-3 and sarafotoxin S6c were bought from The Peptide Institute, Osaka, Japan, and VEGF was from Sigma (Poole, UK). All other reagents were analytical reagent grade obtained from Merck or Fisher. Using an Axopatch 100 amplifier (Axon Instruments, Foster City CA, USA) driven by a CED 1401 interface (Cambridge Electronic Design, Cambridge, UK), cells were clamped at an intracellular holding potential of -60 mV and currents recorded in response to test pulses of amplitude between -100 mV and +100 mV (pulse duration: 200 ms, applied every second) to estimate the cell's current-voltage relation. Off-line analysis of currents was performed using Strathclyde software (generously provided by Dr J. Dempster, University of Strathclyde, UK).

Results

Both VEGF, and as reported earlier [5] ET-3, increased the rate of regrowth of HUVEC after mechanical wounding. The fractional reduction of the damaged area at 18 h due to regrowth in control conditions was 0.236 ± 0.004 (mean \pm

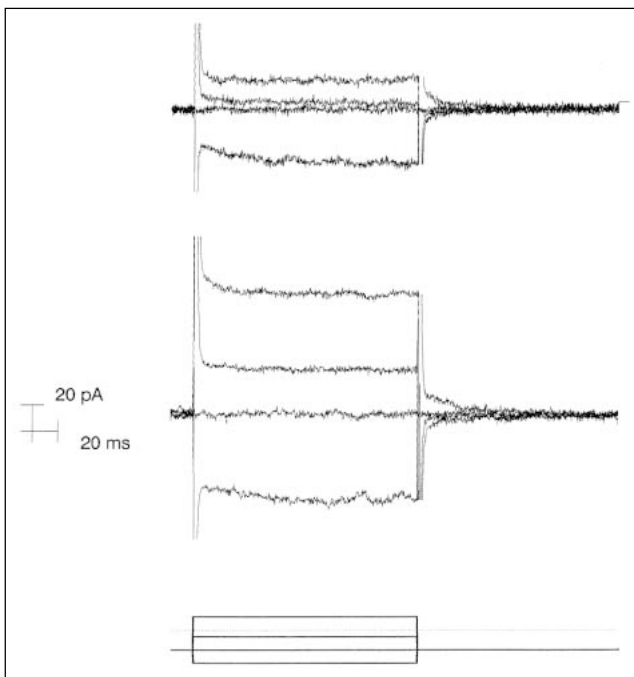


Figure 1: Effect of acute exposure to vascular endothelial growth factor (VEGF) on whole cell currents recorded from human umbilical vein endothelial cells (HUVEC) on the edge of a regrowing boundary. Whole cell currents before (upper traces) and at the end of 10 min in VEGF (20 pmol/L) containing solution (middle traces). From a holding potential of -60mV, brief pulses to -100, -60, -20 and +40mV were applied (pulse protocol shown in bottom trace). Whole cell capacitance 24 pF, series resistance 5.5 M Ω . Currents are shown with residual capacity transients manually erased. Cell 601252.

SEM of three independent experiments, each with quadruplicate assays). In the presence of 20 pmol/L VEGF, the fractional reduction in wound area was 0.321 ± 0.012 and, in the presence of 100 nmol/L ET-3, 0.441 ± 0.02 , both being significantly different from the control value ($p < 0.05$, unpaired t -test). The actual cell numbers for the experiments with ET-3, expressed in thousands of cells, were 22.7 ± 1.4 in control conditions, 31.0 ± 1.1 in 100 nmol/L ET-3.

Whole cell recordings were made from HUVEC, 1–8 h post-wounding. In cells at a regrowing boundary on the edge of a strip of wound, application of either VEGF (20 pmol/L) or ET-3 (100 nmol/L) for 10 min produced a consistent increase in whole-cell current within about 10 min, in more

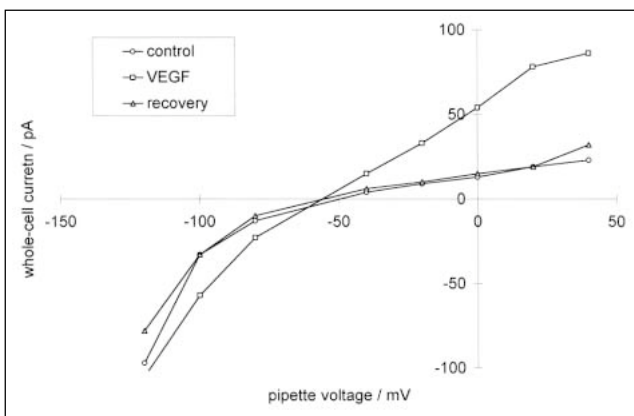


Figure 2: Current voltage relations derived from the same cell as in Figure 1, before (control) and at the end of a 10 min exposure to VEGF (20 pmol/L) then 60 min after VEGF removal (recovery). Note the inward rectifier current present at voltages more negative than -80mV throughout this recording.

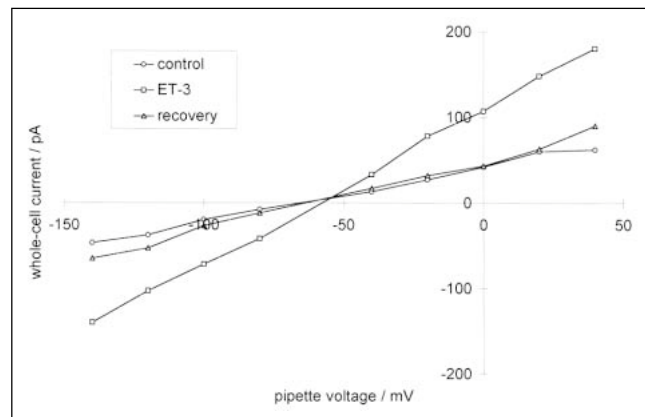


Figure 3: Effect of acute endothelin-3 (ET-3) treatment on whole cell currents recorded from a single HUVEC on the edge of a wound. Current voltage relations are shown before ET-3 application (control), at the end of a 10 min exposure to 100 nmol/L ET-3 after which ET-3 was removed, then 60 min after ET-3 removal (recovery). Cell 511072. Whole cell capacitance 29pF, series resistance 8 M Ω .

than half of the cells studied ($n = 7/11$; see Fig. 1). The currents showed little or no relaxation in the course of a 200 ms pulse and the conductance showed little voltage dependence. Typical current-voltage relations for the peptide-induced currents are shown in Figures 2 and 3. The reversal voltage for the VEGF-induced current was -59 ± 1 mV (mean \pm SD, $n = 4$) and that for the ET-3 induced current was also -59 ± 1 mV ($n = 3$). For both VEGF and ET-3, recovery of the conductance following peptide removal was slow, a typical interval for near-complete recovery being 60 min. When cells at the edge of a regrowing boundary were exposed to the selective ET_B receptor agonist sarafotoxin S6c (40 nmol/L), no such increase in whole cell current was observed (data not shown; $n = 3/3$). ET-3 application (100 nmol/L) induced no electrophysiological effects in three recordings from cells distant from the edge of a wound (0.15–0.20 mm from the regrowing boundary; not shown, $n = 3/3$).

Discussion

Electrophysiological and regrowth-promoting effects of VEGF and ET-3

Since previous work has found rapid electrophysiological effects that correlate with morphological or trophic changes in endothelial cells [10–12] the present study examined whether the effects of ET-3 and VEGF on HUVEC regrowth are accompanied by changes in whole-cell current. It was found that acute application of either VEGF or ET-3, but not sarafotoxin S6c, activated currents in cells on the edge of a wound in HUVEC monolayers, at concentrations similar to those which stimulated regrowth. The lack of effect of sarafotoxin S6c strongly suggests that the ET-3 induced currents observed in the present study are not ET_B receptor dependent and hence are likely to result from ET_A receptor stimulation. Since acceleration of HUVEC regrowth by endothelins is mediated by ET_B receptors [5, 8] the two effects are unlikely to be causally related. Although the pharmacological measurements on regrowth were taken after 18 h, whereas the electrophysiological experiments were carried out less than 8 h after wounding, it is likely that the ET-3-induced currents are not a requirement for ET-3-dependent acceleration of repair [5–8].

Ionic conductances underlying ET-3 and VEGF induced currents

The whole cell currents activated by ET-3, and those activated by VEGF, both had a reversal voltage of -59 mV. This voltage is an indicator of the ionic conductance underlying the current increase, as a conductance that is highly selective for a given ion has a reversal voltage close to the Nernst potential for that ion. The ionic species present here have the following calculated Nernst potentials: K^+ : -101 mV; Cl^- : -50 mV; Mg^{2+} : -27 mV; Na^+ : +53 mV. Calcium, sulphate and gluconate ions are all likely to have a highly positive Nernst potential. The reversal voltage observed here is indicative of a cation conductance that is weakly selective for potassium and, assuming that the major permeant species are K^+ and Na^+ , corresponds to a calculated permeability ratio $p_K/p_{Na} = 3$. The very similar reversal voltages observed with either VEGF or ET-3, clearly different from all the calculated Nernst potentials, suggest that the two peptides may be activating the same channel population, although this cannot be determined with certainty from the present data. An increase in chloride conductance is an unlikely explanation for the currents observed here, since under the present experimental conditions, a chloride selective channel would have a reversal voltage less negative than -50 mV.

Gap junctional conductance pathways between the cell under study and its neighbours, manifested as "pseudo-cation" currents, have been observed in bovine pulmonary aortic endothelial cells [13]. This is unlikely to explain the present data, since first, the pseudo-cation currents were found in all cells with neighbours and never in solitary cells [13]; second, the pseudo-cation currents, if present, were present throughout recordings and their reversal voltage shifted with time [13]; finally, the reversal voltage of the gap junctional conductance would be expected to vary, depending on the resting voltage of neighbouring cells and the degree of electrical access between them. None of these three phenomena was observed here.

Though endothelial electrophysiology has been studied extensively (see [14]) there are only a few previous reports of endothelin-induced currents in endothelial cells. Both ET-1 and ET-3 induce cation currents in rat brain endothelial cells that somewhat resemble those observed here [15] while in bovine pulmonary artery endothelial cells, ET-1 activates a calcium-permeable, non-selective, cation channel [16, 17]. In contrast, the potassium channels that are activated by either muscarinic receptors or mechanical stress in bovine aortic endothelial cells [18, 19] display strong inward rectification and K^+ selectivity. There have been no previous reports of VEGF-induced ionic currents, though VEGF causes rapid elevation in intracellular calcium concentration in HUVEC, possibly by activating an inositol phosphate pathway ($EC_{50} = 0.4$ pmol/L) [20].

In conclusion, VEGF and ET-3 both induce cation currents in regrowing HUVEC at concentrations which have been shown to promote growth. The relationship between these electrophysiological responses and vascular repair remains to be determined.

Acknowledgements

The authors are grateful to Drs. S. B. Hladky, J. Dempster and G. T. H. Ellison for advice and access to resources. Financial support was provided by the Biotechnology and Biological Science Research Council of Great Britain (studentship for ELKS) and the Medical Research Council of Great Britain (studentship for HL).

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