Supplementary Methods

Primer sequences used in the RT-PCR analyses

The primer oligonucleotide sequences used in the RT-PCR assays were: 5'-GAG CAC TAC GCA GTC AGT CG-3' (sense) and 5'-TTT TCC TTG GCT GGA GAG G-3' (antisense) for $S1P_1$ (Genbank accession number NM_001400.4); 5'-CAT TGT TTT GAA CTA GGT GGA CA-3' (sense) and 5'-CAA GCC TTT TTG GGG AAT TT-3' (antisense) for $S1P_3$ (Genbank accession number NM_005226.2); 5'-CCG CTC AAG CCA CGC TGA CA-3' (sense) and 5'-CGG CTG CGC CAC GTG TAG AT-3' (antisense) for $S1P_2$ (Genbank accession number NM_004230.3); 5'-GAA CAT TTG GGA AAT CTC TTG C-3' (sense) and 5'-CGG AAG AAC AAT GTA GTC TTT GC-3' (antisense) for VEGFR2 (Genbank accession number NM_002253.2); and 5'-ACC ACA GTC CAT GCC ATC AC-3' (sense) and 5'-TCC ACC ACC CTG TTG CTG TA-3' (antisense) for GAPDH (Igarashi *et al*, 2007), respectively.

cAMP assay

HUVEC in a 6 well plate were treated with various agents in the presence of 500 μM of isobutylmethylxanthine. They were then scraped into 200 μL of 100 μM HCl and subjected to enzyme-linked immunosorbent assay using a commercially available assay kit (Cayman, Ann Arbor, MI, USA) following the instruction.

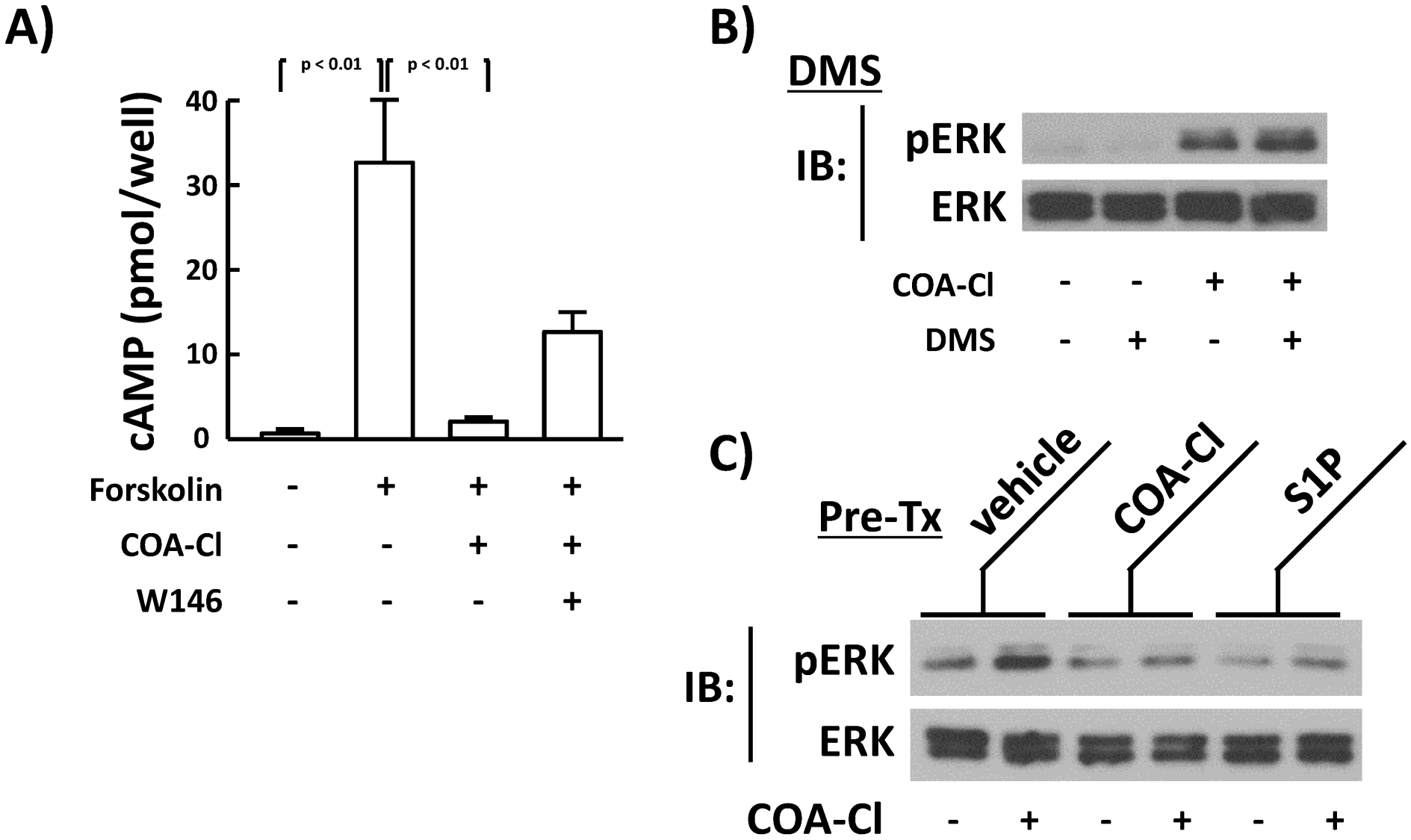
Supplementary Figure Legends

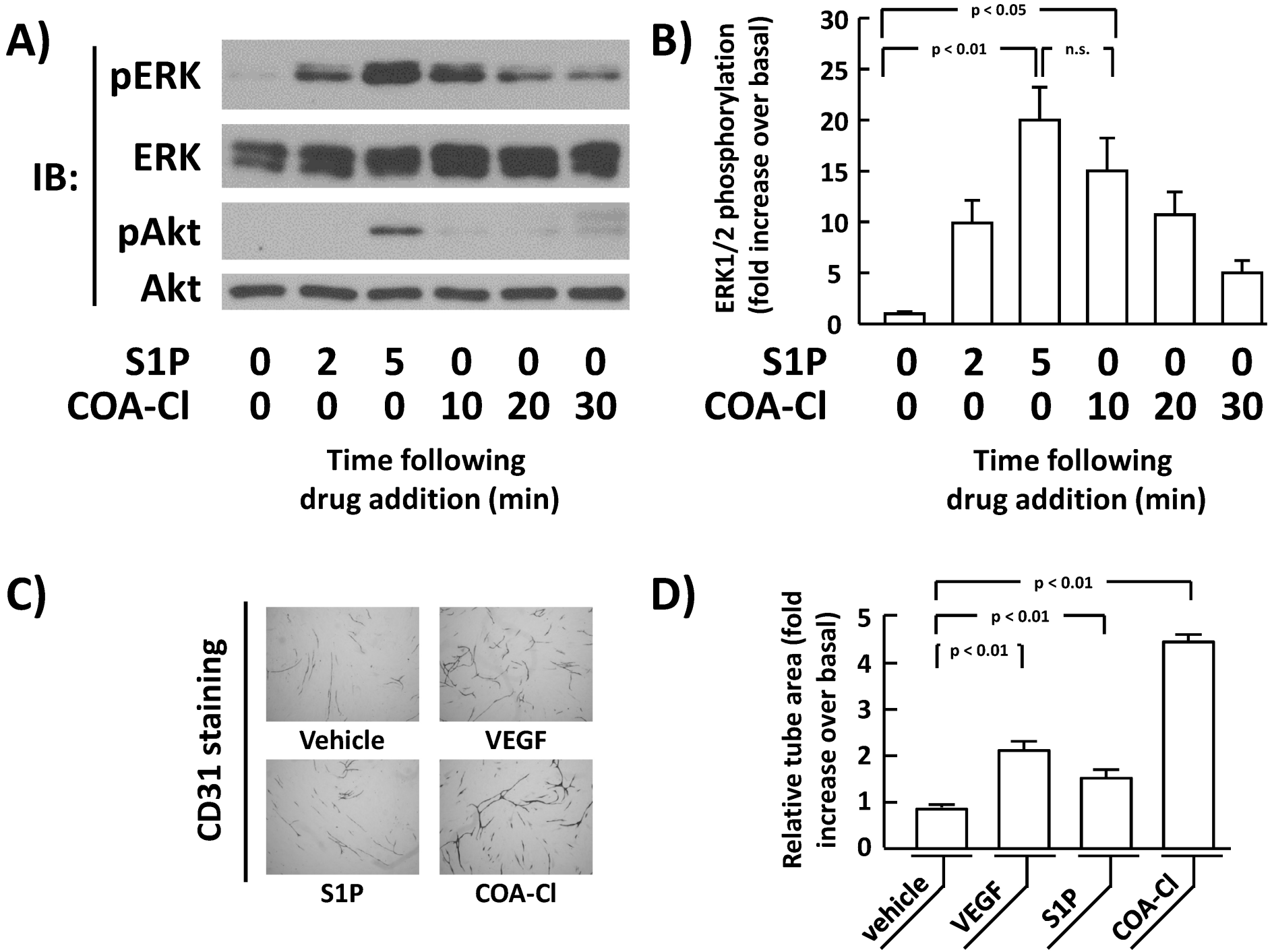
Figure S1. Pharmacological characterization of HUVEC responses to COA-Cl.

Panel A shows the results of cAMP assay. HUVEC were treated for 30 min with forskolin (25 μM) or W146 (10 μM), followed by COA-Cl (100 μM for 10 min), as indicated. They were then subjected to cAMP determination. n = 3. Panel B demonstrates the results of immunoblot (IB) analyses, in which HUVEC were treated with DMS (10 μM for 30 min), an inhibitor of sphingosine kinase, followed by COA-Cl (100 μM for 10 min). Cells were harvested and were subjected to IB using antibodies directed to phospho- and total-ERK1/2, as above. n = 3. Panel C shows the results of receptor desensitization assay. Some cells had been pre-treated ("Pre-Tx") with COA-Cl (100 μM), S1P (100 nM) or vehicle for 60 min prior to COA-Cl (100 μM for 10 min). n = 3.

Figure S2. Comparison of COA-Cl actions with S1P in HUVEC.

Shown in Panel A are the results of immunoblot assays using the cell lysates derived from HUVEC treated with S1P (1 μM) or with COA-Cl (100 μM) for the times indicated, performed in an identical culture batch. They were subjected to IB for phospho- and total-ERK1/2 as above. Blots were also re-probed with antibodies directed to phospho- and total-Akt, as well. The degrees of ERK1/2 phosphorylation were quantified and are summarized in a graph shown in the Panel B. n = 4. Shown in Panels C and D are the results of tube formation assays, in which cells were treated with vehicle, VEGF (10 ng/mL), S1P (1 μM), or COA-Cl (100 μM), performed in the identical cellular preparations. n = 5.


A)
p < 0.01
p < 0.01
cAMP (pmol/well)
40
30
20
10
0
Forskolin - + + +
COA-Cl - - + +
W146 - - - +
B)
DMS
IB:
pERK
ERK
COA-Cl - - + +
DMS - + - +
C)
Pre-Tx
vehicle
COA-Cl
S1P
IB:
pERK
ERK
COA-Cl - + - + - +


A)
IB:
pERK
ERK
pAkt
Akt
S1P 0 2 5 0 0 0
COA-Cl 0 0 0 10 20 30
Time following drug addition (min)
B)
ERK1/2 phosphorylation (fold increase over basal)
p < 0.05
p < 0.01
n.s.
30
25
20
15
10
5
0
S1P 0 2 5 0 0 0
COA-Cl 0 0 0 10 20 30
Time following drug addition (min)
C)
CD31 staining
Vehicle
VEGF
S1P
COA-Cl
D)
Relative tube area (fold increase over basal)
p < 0.01
p < 0.01
p < 0.01
5
4
3
2
1
0
vehicle
VEGF
S1P
COA-Cl