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Compound C inhibits in vitro angiogenesis and ameliorates thrombin-induced endothelial barrier failure

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ABSTRACT

Compound C (comp. C) is a cell-permeable pyrrazolopyrimidine derivative and widely used as adenosine monophosphate-activated protein kinase (AMPK) inhibitor to characterise the role of AMPK in various physiological processes. However, its AMPK-independent effects have also been reported. In the present study we investigated the effects of moderate dose (1–10 μM) comp. C on endothelial cell (EC) proliferation, in vitro angiogenesis, and endothelial barrier function. Comp. C was unable to inhibit AMPK phosphorylation (activation) induced by metformin and A-769662 in ECs even at concentration of 10 μM. At lower concentration (1 μM), comp. C inhibited and potentiated the inhibitory effects of metformin and A-769662 on EC proliferation, migration, tube formation, and sprouting without inducing apoptosis. However, at higher concentration (10 μM), it strongly induced apoptosis as measured by enhanced caspase 3/7 activity. Moreover, comp. C antagonised thrombin-induced EC hyperpermeability accompanied by activation of Rac1 and strengthening of adherens junctions (AJs). This EC barrier protective effect was not affected by the presence of AMPK activators. The data of the present study demonstrate that long-term treatment of ECs with low concentration comp. C inhibits EC proliferation and angiogenesis without induction of apoptosis. While short-term incubation antagonises thrombin-induced EC hyperpermeability presumably via Rac1-dependent strengthening of AJs. Furthermore, higher concentration of comp. C (10 μM or above) is toxic for ECs and warns that this agent should be used with caution to demonstrate the AMPK-mediated effects.

1. Introduction

Adenosine monophosphate-activated protein kinase (AMPK) is a key regulator of cellular and whole body energy homoeostasis (Hardie et al., 2012). Additionally, in endothelial cells (ECs) it is involved in the regulation of several key cellular processes like apoptosis (Liu et al., 2010), autophagy (Yan et al., 2010), angiogenesis (Nagata et al., 2003), and differentiation (Zhu et al., 2011). AMPK exists as a heterotrimeric complex consisting of a catalytic α subunit and regulatory β and γ subunits (Nagata and Hirata, 2010). Activation of AMPK requires phosphorylation of the α subunit at Thr172 by upstream kinases including LKB1 and Ca^{+2}-dependent CaMKKβ. Pharmacologically, AMPK can be activated with AICAR, metformin, and A-769662 (Göransson et al., 2007; Nagata and Hirata, 2010). In order to analyse the role of AMPK in different cellular process, these agents serve as important tools to specifically activate the AMPK related signalling (Nagata and Hirata, 2010). Likewise, AMPK can be inhibited pharmacologically using (6-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-pyridin-4-yl-pyrazolo [1,5-a]pyrimidin) also known as compound C (comp. C) or dorsomorphin, which is a cell permeable pyrrazolpyrimidine derivative and acts as an ATP-competitive inhibitor of AMPK in vitro (Zhou et al., 2001).

Although, in vitro non-cellular kinase assays suggest that comp. C inhibits AMPK activity with IC50 ranging from 0.1 to 0.2 μM, a minimal 40 μM concentration is required in culture medium to inhibit AMPK activity in cells (Bain et al., 2007). Thus, in most cell culture studies it is used at concentration range of 10–100 μM (Zhou et al., 2001; Piwkowska et al., 2012). Accumulating data

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report AMPK-independent actions of comp. C albeit, comp. C is widely used to verify the role of AMPK in various (patho)-physiological processes. For example, comp. C inhibits bone morphogenetic protein 1 (BMP1) receptors (Yu et al., 2008), stabilises hypoxia-inducible factor-1α (Emerling et al., 2007), and inhibits several other kinases in an AMPK-independent manner (Bain et al., 2007). Moreover, comp. C also inhibits the growth of both rodent and human glioma cell lines (Vucicevic et al., 2009; Liu et al., 2014) and rodent smooth muscle cells (SMC) (Peyton et al., 2011) via AMPK-independent mechanisms.

Given the reported AMPK-independent actions of comp. C in mammalian cells of different origin, the present study investigated the effects of comp. C on EC proliferation, angiogenesis, and barrier function at concentration range well below required to inhibit AMPK activity. The study was performed in well-established cell culture model of human umbilical vein endothelial cells (HUVECs).

2. Materials and methods

2.1. Materials

HRP-conjugated anti-mouse IgG, and rabbit IgG antibodies were from Amersham Biosciences (Heidelberg, Germany); matrigel was from BD (Heidelberg, Germany); anti phospho ACC (Ser9), anti phospho AMPK (Thr172), anti GAPDH were from Cell Signaling Technologies (Danvers, USA); compound C and metformin were from Enzo life science (Loerrach, Germany); Pierce® ECL solution was from Fischer Scientific (Niederlassung Nidderau, Germany); Complete® protease inhibitor cocktail was from Roche (Mannheim, Germany); human thrombin was from Sigma (Steinheim, Germany); A-769662 was from Tocris (Bristol, UK); ThinCert® polycarbonate membrane filters (24-mm round) were from Greiner Bio-One (Frickenhausen, Germany). All other chemicals were of the best available quality, usually analytical grade.

2.2. Cell culture

The study conforms to the principles outlined in the "Declaration of Helsinki" (*Cardiovascular Research* 1997; 35: 2–3). HUVEC were isolated and cultured as described previously (Aslam et al., 2012) in complete EC culture medium (Cat #C-22010; PromoCell, Heidelberg, Germany) and used at passage 1. HUVEC were cultured in 6-well plates for Western blotting, 96-well plates for caspase assay, 6-well filter inserts for permeability assay, 12-well plates for migration assay, and 10 cm culture dishes for pulldown assay.

2.3. Experimental protocols

The basal medium used in incubations was modified Tyrode's solution (composition in mM: 150 NaCl, 2.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.0 $CaCl_2$, and 30.0 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4, 37 °C). Agents were added as indicated. Stock solutions of metformin, sodium fluoride, and thrombin were prepared in basal medium and that of A-769662 and comp. C in warm DMSO. Appropriate volumes of these solutions were added to the cells yielding final solvent concentrations $< 0.1\%$ (vol/vol). Where combination of drugs was used, comp. C was added 30 min before adding the other agents. The same final concentrations of basal medium were included in all respective control experiments. The experiments for permeability measurement were performed in the presence of 2% FCS while for that for Western blot analysis were performed in serum free Tyrode's solution. All the angiogenesis and proliferation assays were performed in complete EC growth medium containing 2% FCS, unless otherwise stated.

In a set of pilot experiments time–response relationships were determined to find the optimal time for AMPK activation and this time point was used in further experiments (Thrombin 10 min, A-769662 and metformin 60–180 min).

2.4. Caspase 3/7 assay

HUVECs were seeded at a density of 5×10^3/well in a 96-well cell culture plate. After 24 h the cells were treated with comp. C for next 24 h or 48 h and caspase 3/7 activity was measured using fluorescence caspase 3/7 kit (Promega, Germany) according to manufacturer's instructions. Fluorescence was measured using Infinite 200® ELISA reader (Tecan, Austria).

2.5. Cell proliferation

HUVECs were seeded at a density of 10^5 in 12-well cell culture plates. After 24 h the cells were treated with different pharmacological agents as mentioned in for another 24 h. The cells were then trypsinised and counted manually using a Neubauer cell chamber.

2.6. Wound healing assay

HUVEC were seeded at a density of 10^5 per well in a 12-well plate with a silicon insert (2×5 mm^2) in the middle of the plate. After 48 h, when ECs established a confluent monolayer, the silicon insert was removed to create a wound. Cells were treated with respective pharmacological agents and the size of wound closure was documented using an inverted microscope connected with a CCD camera (Olympus). The migration area was quantified by using Cell D software (Olympus). Each experiment was performed in triplicate and the average was calculated from at least 5 different places in each well.

2.7. Cell sprouting assay

Cell sprouting assay was described by DeBusk et al. (2004). Briefly, HUVECs were grown to 70–75% confluence, trypsinised, and mixed with EGM with 20% FCS and 0.2% methyl cellulose. The cell mixture was then incubated overnight at 37 °C in uncoated, round bottom, sterile 96-well Petri culture dishes (greiner bio-one). The cells formed spheres around the methyl cellulose particles. The spheres were collected via centrifugation at 800 RPM and resuspended in EGM with 20% FCS and 0.2% methyl cellulose. Equal parts of cell sphere suspension and rat tail collagen I solution (2 mg/ml) plus 1% NaOH solution (in PBS) were then combined and plated in 24-well tissue culture plates. The plates were then incubated at 37 °C for 30 min, overlaid with EGM with 20% FCS containing different pharmacological agents or DMSO, and then incubated at 37 °C overnight in a cell culture incubator. After 24 h, cell sprouts were photographed and analysed.

2.8. Tube formation assay

Ice cold matrigel solution (300 µl) was added to each well in a 24-well plate on ice and allowed to gel for 30 min at 37 °C. HUVECs were trypsinised and added (12×10^4/well) on top of the matrigel. Different pharmacological agents were pre-mixed with the cells in medium in respective concentration before adding cells to the matrigel. Cells were photographed after 8 h. The total number of tubes from the nodes of ECs was quantified using Cell D software (Olympus).