R1: Inhibition of TNFα-stimulated monocyte adhesion to human aortic endothelial cells by AMP-activated protein kinase

Salt: Running title: AMPK and monocyte adhesion

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ABSTRACT

Objective: Pro-atherosclerotic adhesion of leukocytes to the endothelium is attenuated by NO. As AMP-activated protein kinase (AMPK) regulates endothelial NO synthesis, we investigated the modulation of adhesion to cultured human aortic endothelial cells (HAECs) by AMPK.

Methods and Results: HAECs incubated with the AMPK activator, AICAR or expressing constitutively active AMPK demonstrated reduced TNFα-stimulated adhesion of pro-monocytic U-937 cells. Rapid inhibition of TNFα-stimulated U-937 cell adhesion by AICAR was NO-dependent, associated with unaltered cell surface adhesion molecule expression and reduced MCP-1 secretion by HAECs. In contrast, inhibition of TNFα-stimulated U-937 cell adhesion by prolonged AMPK activation was NO-independent and associated with reduced cell surface adhesion molecule expression.

Conclusions: AMPK activation in HAECs inhibits TNFα-stimulated leukocyte adhesion by a rapid NO-dependent mechanism associated with reduced MCP-1 secretion and a late NO-independent mechanism whereby adhesion molecule expression, in particular E-selectin, is suppressed.

CONDENSED ABSTRACT

We investigated the functional effects of AMPK activation in cultured human endothelial cells. Stimulation of AMPK inhibited TNFα-stimulated monocyte adhesion by two distinct mechanisms; a rapid NO-dependent mechanism associated with a reduction in chemokine release and a late NO-independent mechanism whereby adhesion molecule expression is suppressed.

Recent studies have demonstrated AMPK-activated protein kinase (AMPK) to be a key regulator of NO synthesis in cultured endothelial cells in response to stimuli including AICAR [1,2]. The functional effects of AMPK-mediated NO production in the endothelium remain poorly characterized, however. Endothelial NO inhibits leukocyte adhesion and the expression of ICAM-1, VCAM-1 and E-selectin in response to pro-inflammatory stimuli such as TNFα [3]. AICAR has recently been reported to reduce TNFα-stimulated monocyte adhesion and mRNA expression of ICAM-1, VCAM-1 and E-selectin in cultured endothelial cells and postischemic leukocyte rolling and adhesion in mice [4,5]. However, these studies did not address whether the effects of AICAR are mediated by NO and/or AMPK, as AICAR has been demonstrated to have AMPK-independent effects [6]. We have, therefore, investigated the molecular mechanisms that underlie reduced monocyte adhesion in response to AICAR.

METHODS

Cell systems and infection

HAECs, cultured as described previously [2], were infected with recombinant adenoviruses expressing constitutively active (Ad.AMPK-CA) mutant AMPK, dominant negative (Ad.AMPK-DN) mutant AMPK or control adenoviruses (Ad.control) as described previously [2].

Monocyte adhesion and chemokine secretion assay

To ensure that observed effects of AMPK activation were specific to HAECs, HAEC monolayers were washed thoroughly with serum-free medium prior to overlay with U-937 cells or chemokine assay. Adhesion was assessed microscopically and chemokine secretion by immunoassay.

Flow cytometry

Cell surface E-Selectin, ICAM-1 and VCAM-1 expression were quantified using anti–VCAM-1, anti-ICAM-1 or anti-E-Selectin antibodies and FITC- or phycoerythrin-labeled secondary antibodies.

For details, see the supplementary materials.

RESULTS

Incubation of HAECs with AICAR reduced TNFα-stimulated U-937 cell adhesion in a time- and dose-dependent manner without affecting basal U-937 cell adhesion (Figure 1A and 1C, Supplemental Figure I) or HAEC viability as assessed by Annexin V staining (data not shown). Infection with Ad.AMPK-CA completely abrogated TNFα-stimulated U-937 cell adhesion, without altering basal adhesion (Figure 1B). Preincubation of HAECs with the eNOS inhibitor, L-NAME, abrogated the rapid (45-90min) inhibition of TNFα-stimulated U-937 cell adhesion by AICAR, but had no effect on inhibition of adhesion in response to long term (120-360min) AICAR treatment or Ad.AMPK-CA (Figure 1A and 1B). Similar effects were observed after siRNA-mediated downregulation of eNOS (Supplemental Figure II). Infection with Ad.AMPK-DN attenuated AMPK activity (Supplemental Figure III) and the inhibition of TNFα-stimulated U-937 cell adhesion by AICAR (Figure 1C).

Incubation of HAECs with AICAR (240min) or infection with Ad.AMPK-CA inhibited TNFα-stimulated cell surface expression of ICAM-1, VCAM-1 and E-selectin yet incubation with AICAR for 45min did not affect adhesion molecule expression (Figure 2A). Infection with Ad.AMPK-DN reversed AICAR-mediated inhibition of E-selectin expression (Figure 2B) and partially reversed inhibition of ICAM-1 and VCAM-1 expression (Supplemental Figure IV). L-NAME did not affect expression of adhesion molecules (Figure 2B and Supplemental Figure IV).

As acute incubation with AICAR reduced U-937 cell adhesion independent of HAEC adhesion molecule expression, we reasoned AICAR may inhibit chemokine secretion by HAECs. Incubation of HAECs with AICAR for 45 min significantly reduced TNFα-stimulated MCP-1 secretion, an effect partially abrogated by co-incubation of HAECs with L-NAME (Figure 2C) or infection with Ad.AMPK-DN (Figure 2D).

DISCUSSION

The central finding of this study is that incubation of HAECs with AICAR markedly reduces TNFα-stimulated monocyte adhesion in a biphasic and AMPK-dependent manner. These data suggest that the rapid, adhesion molecule-independent effects of AICAR are mediated by AMPK-stimulated NO synthesis reducing secretion of MCP-1 and potentially secretion of other chemokines by HAECs in response to TNFα. Prolonged incubation with AICAR reduces TNFα-stimulated E-selectin expression in an AMPK-dependent, NO-independent manner, whereas AICAR reduces TNFα-stimulated ICAM-1 and VCAM-1 expression by a mechanism only partially dependent on AMPK. A recent study reported that AICAR reduced postischemic leukocyte adhesion in an eNOS-independent manner, yet leukocyte rolling in response to prolonged AICAR treatment was eNOS-dependent [5]. The differences between the current study and these data may reflect the different species studied and lack of assessment of the AMPK-dependence and site of action of AICAR *in vivo*.

The evidence presented in this study clearly implicates AMPK activation as a potential anti-atherogenic mechanism. Atherosclerosis is commonly associated with type 2 diabetes and insulin resistance and we propose that AMPK, in addition to its potential to correct the metabolic defects present in type 2 diabetes and insulin resistance [7] is an attractive candidate therapeutic target for reducing associated atherogenesis.

SOURCES OF FUNDING

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FIGURE LEGENDS

Figure 1. AMPK-mediated inhibition of U-937 cell adhesion to HAECs.

The effect of A) AICAR or B) Ad.AMPK-CA on TNFα-stimulated U-937 cell adhesion to HAECs in the presence or absence of L-NAME. C) AMPK-dependence of AICAR-mediated inhibition of adhesion.

Figure 2. Effect of AMPK activation on adhesion molecule expression and MCP-1 secretion.

Effect of AICAR or Ad.AMPK-CA on TNFα-stimulated A) HAEC adhesion molecule expression or C) MCP-1 secretion. AMPK-dependence of AICAR-mediated inhibition of B) adhesion molecule expression or D) MCP-1 secretion.

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FIGURE 1

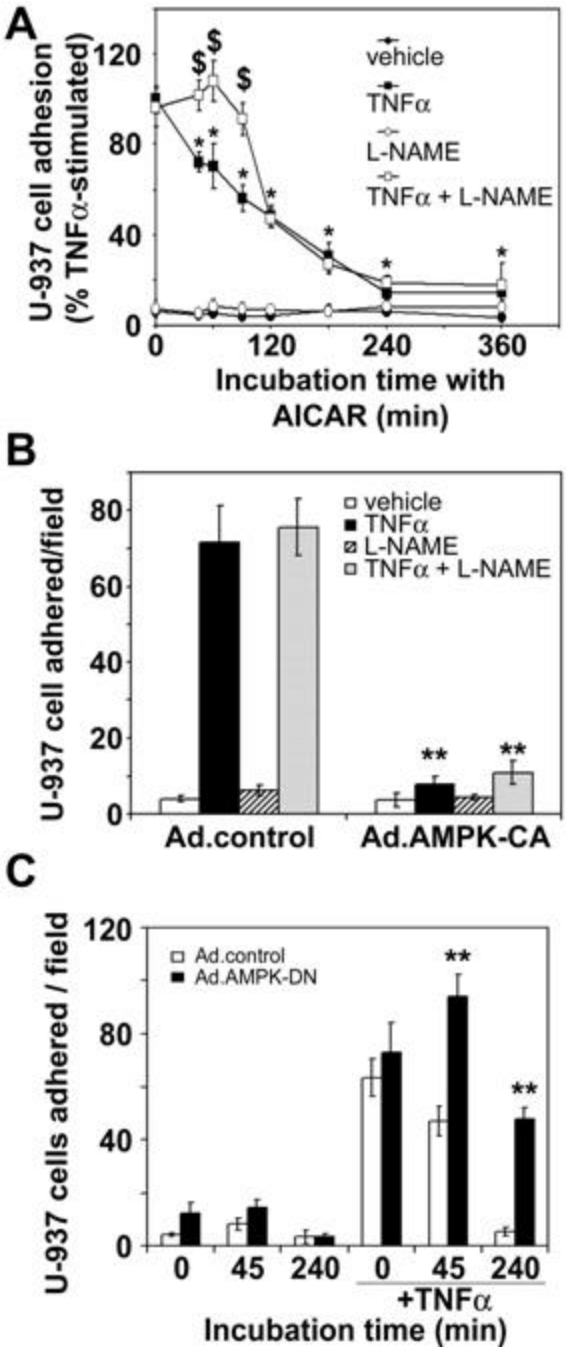
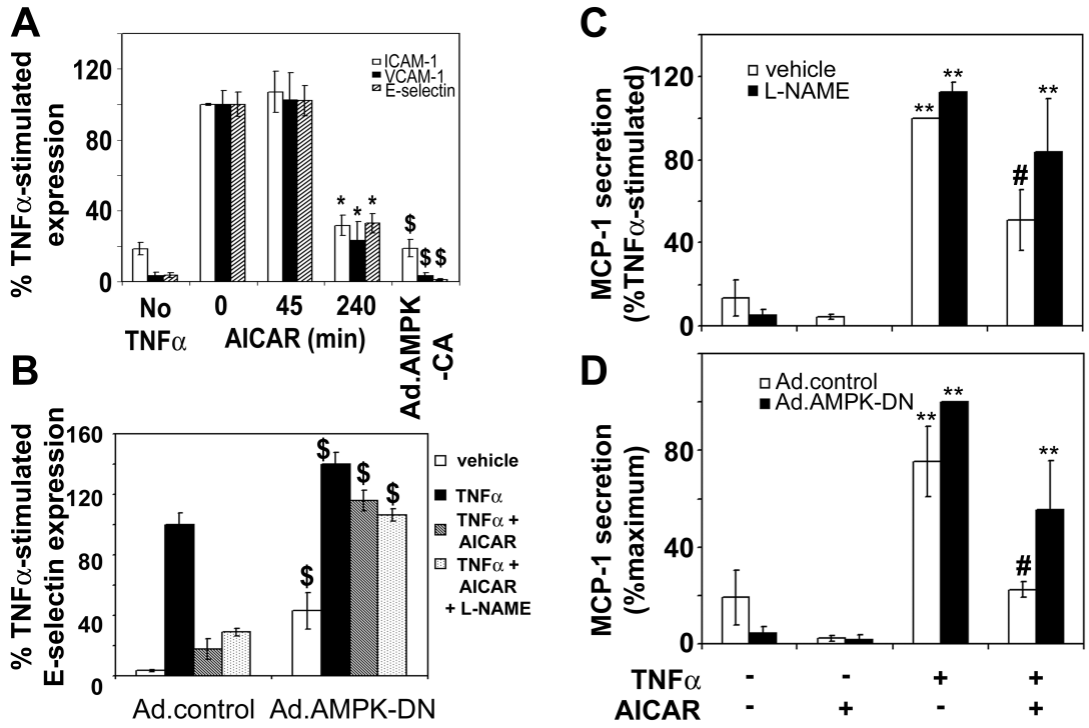

A
U-937 cell adhesion (% TNFα-stimulated)
120
80
40
0
$
$
$
*
*
*
*
*
*
*
vehicle
TNFα
L-NAME
TNFα + L-NAME
0
120
240
360
Incubation time with AICAR (min)
B
U-937 cell adhered/field
80
60
40
20
0
vehicle
TNFα
L-NAME
TNFα + L-NAME
**
**
Ad.control
Ad.AMPK-CA
C
U-937 cells adhered / field
120
80
40
0
Ad.control
Ad.AMPK-DN
**
**
0
45
240
0
45
240
+TNFα
Incubation time (min)

FIGURE 2


A
% TNFα-stimulated expression
ICAM-1
VCAM-1
E-selectin
No TNFα
0
45
240
AICAR (min)
Ad.AMPK -CA
B
% TNFα-stimulated E-selectin expression
vehicle
TNFα
TNFα + AICAR
TNFα + AICAR + L-NAME
Ad.control
Ad.AMPK-DN
C
MCP-1 secretion (%TNFα-stimulated)
vehicle
L-NAME
D
MCP-1 secretion (%maximum)
Ad.control
Ad.AMPK-DN
TNFα
AICAR

SUPPLEMENTARY MATERIALS AND METHODS

Materials

Oligofectamine was supplied by Invitrogen Ltd (Paisley, Renfrewshire, UK). Mouse anti-VCAM-1, mouse anti-ICAM-1, FITC- and phycoerythrin-labeled anti-mouse antibodies were obtained from Abcam (Cambridge, UK). Mouse anti-E-selectin antibodies were obtained from Serotec (Oxford, UK). Dharmacon ON-TARGETplus SMARTpool siRNA targeted to eNOS, GAPDH and scrambled control siRNA were obtained from Thermo Fisher Scientific Biosciences UK (Cramlington, Northumberland, UK). All other reagents were from sources described previously [1-3].

Cell Culture and recombinant AMPK adenoviruses

HAEC and U-937 cells were cultured as described previously [1,2]. HAECs were infected with 20-80 pfu/cell recombinant adenoviruses expressing constitutively active (Ad.AMPK-CA) mutant AMPK, dominant negative (Ad.AMPK-DN) mutant AMPK or control adenoviruses (Ad.control: adenoviruses expressing GFP alone for experiments with Ad.AMPK-CA or lacking any exogenous gene for experiments with Ad.AMPK-DN), propagated and purified as described previously [1,2,4].

Transfection of HAECs with siRNA

Serum-free endothelial cell culture medium (300 μl) containing 0.25% (v/v) oligofectamine and 0.17 μM siRNA was added to each well of HAECs grown to near confluence on 24-well tissue culture plates. After 5 h, 250 μl of large vessel endothelial cell medium was added. After 24 h, medium was removed and the transfection procedure repeated. After a further 24 h, cell lysates were prepared or U-937 adhesion assessed.

Preparation of HAEC lysates

HAEC lysates were prepared and proteins resolved by SDS-PAGE and western blotting as described previously [1-3].

Monocyte Adhesion Assay

HAECs were infected with recombinant AMPK adenoviruses or transfected with siRNA, if desired, for 24 h or 48 h respectively prior to adhesion assay. Adhesion of U-937 cells was assessed as described previously [2]. As HAEC monolayers were washed thoroughly with serum-free medium prior to overlay with U-937 cells, the U-937 cells are not exposed to adenoviruses, TNFα, L-NAME or AICAR using this protocol, ensuring that observed effects are HAEC-mediated and not a result of altered U-937 function.

Flow cytometry for cell surface VCAM, ICAM and E-Selectin expression

HAECs were grown to confluence on 12-well tissue culture plates and infected with recombinant AMPK adenoviruses, if desired, for 24 h with subsequent stimulation as described in figure legends. Cells were gently dislodged (0.025% (w/v) trypsin, 2 mM EDTA in PBS), neutralized with complete cell culture medium and re-suspended in 100 µL PBS supplemented with 1% (w/v) BSA (PBS-BSA). After incubation with saturating concentrations of anti–VCAM-1, anti-ICAM-1 or anti-E-Selectin antibodies for 1 h, cells were washed three times in PBS-BSA, incubated with saturating concentrations of FITC- or phycoerythrin-labeled secondary antibody for 1 h, washed three times in PBS-BSA and fixed with 0.5% (w/v) paraformaldehyde in PBS. Cell surface adhesion molecule expression was evaluated by flow cytometry in a FACscan II (Becton

Dickinson, Oxford, UK). 10^4 cells were analyzed per sample and data are expressed relative to the percentage of adhesion molecule positive TNFα-stimulated cells. Control experiments were performed in cells incubated with secondary antibodies alone.

Analysis of conditioned medium from HAECs for chemotactic potential

Conditioned medium was obtained from HAECs incubated for 1h as described for monocyte adhesion analysis (after thorough washing to remove any adenoviruses, TNFα, AICAR or L-NAME). Migration of U-937 cells was determined using a 48-well Boyden chamber, (8 µm pore size). Cells were resuspended (10^6 cells/ml) in RPMI 1640 supplemented with 0.1% (w/v) BSA. Cell suspension (50 µl, 0.5 x 10^5 cells) was added to the top chamber of the Boyden chamber and 29 µl of conditioned media was added to the lower chamber. The chamber was incubated at 37°C for 2 h and migrated cells were collected from the lower well and counted in urinalysis glasstic slides (Stratagene, Cambridge, UK). Chemokines were assayed in conditioned medium using a BioSource human chemokine multiplex bead immunoassay kit and a Luminex 100™ detection system, testing for the presence of human chemokines MCP-1, MCP-2, MCP-3, MIP-1a, MIP-1ß, Eotaxin, GRO, RANTES, IP-1 and MIG.

Statistical analysis

Unless stated otherwise, results are expressed as the means ± S.D. Statistically significant differences were determined using an independent-samples Student's *t* test, with $p < 0.05$ as significant.

SUPPLEMENTARY RESULTS

Dose-dependence of AICAR-mediated inhibition of U-937 cell adhesion to HAECs.

Incubation of HAECs with AICAR for 45 min significantly inhibited TNFα-stimulated U-937 cell adhesion at a concentration of 0.5 mM and was maximally effective at a concentration of 2 mM (Supplemental Figure I).

NO-dependence of AICAR-mediated inhibition of U-937 cell adhesion to HAECs.

As demonstrated in Figure 1A, preincubation of HAECs with L-NAME abrogated the rapid inhibition of TNFα-stimulated U-937 cell adhesion by 45-90 min incubation with AICAR, but had no effect on inhibition of TNFα-stimulated adhesion in response to long term AICAR treatment (120-360 min). To support the temporal nature of this NO-dependent action of AICAR, we used siRNA targeted to eNOS to downregulate eNOS. Transfection of HAECs with siRNA targeted to eNOS significantly downregulated eNOS protein expression compared to scrambled control siRNA and attenuated the rapid inhibition of TNFα-stimulated adhesion in response to AICAR (Supplemental Figure II). Transfection with siRNA targeted to eNOS was without effect on either unstimulated U-937 cell adhesion or inhibition of TNFα-stimulated adhesion in response to long term AICAR treatment.

AMPK-dependence of AICAR-mediated inhibition of U-937 cell adhesion to HAECs.

To ensure that downregulation of AMPK activity occurred upon infection with Ad.AMPK-DN, phosphorylation of the AMPK substrate, acetyl-CoA carboxylase (ACC) was assessed by western blotting of cell lysates. As described previously, infection of HAECs with Ad.AMPK-DN prevented AICAR-stimulated phosphorylation of the AMPK substrate ACC (Supplemental Figure III) [1,3]. Infection with Ad.AMPK-DN had no effect on basal U-937 cell adhesion compared to cells infected with control adenoviruses (Figure 1C).

Effect of AICAR on adhesion molecule expression.

TNFα markedly stimulated HAEC surface expression of ICAM-1, VCAM-1 and E-selectin. Neither AICAR nor Ad.AMPK-CA had any significant effect on basal cell surface expression of adhesion molecules (data not shown). Infection of HAECs with Ad.AMPK-DN did not significantly alter basal or TNFα-stimulated E-selectin, ICAM-1 or VCAM-1 cell surface expression (Figure 2B and Supplemental Figure IV). Infection with Ad.AMPK-DN caused a significant yet modest reduction in the inhibition of TNFα-stimulated ICAM-1 or VCAM-1 cell surface expression by AICAR (Supplemental Figure IV).

Effect of AICAR on U-937 cell chemotaxis and HAEC chemokine secretion.

As acute incubation with AICAR altered U-937 cell adhesion independent of HAEC adhesion molecule expression, we initially determined whether migration of U-937 cells was reduced towards conditioned media from AICAR-treated HAECs. In these experiments, conditioned media was collected from HAECs subsequent to infection with Ad.control or Ad.AMPK-DN and stimulation in the presence or absence of TNFα and/or L-NAME for 6 h and AICAR for 45 min. Since the HAECs were thoroughly washed to remove TNFα, L-NAME and AICAR prior to collection of conditioned medium, these had no effect on migration directly. U-937 cell migration was increased towards conditioned media from TNFα-stimulated HAECs (Supplemental Figure V). Conditioned media from HAECs incubated with AICAR for 45 min in the presence or absence of TNFα elicited less U-937 cell migration compared to conditioned media from HAECs incubated in the absence of AICAR (Supplemental Figure V). L-NAME completely abrogated the effect of AICAR preincubation on basal and TNFα-stimulated chemokine secretion by HAECs as assessed by U-937 cell migration (Supplemental Figure V). Furthermore, infection of HAECs with Ad.AMPK-DN completely attenuated

AICAR-stimulated inhibition of basal migration and markedly, but partially reduced AICAR-mediated inhibition of migration towards conditioned medium from TNFα-stimulated HAECs (Supplemental Figure V).

In addition to analysis of MCP-1 secretion (Figures 2C and 2D), secretion of 9 other chemokines (MCP-2, MCP-3, MIP-1a, MIP-1ß, Eotaxin, GRO, RANTES, IP-1 and MIG) was assessed simultaneously in conditioned medium obtained under identical conditions to those in Supplemental Figure V. HAECs secreted quantifiable concentrations of MCP-1 (approximately 4ng/hr/10^6 HAECs under stimulated conditions), MCP-2, Eotaxin, IP-10 (all approximately 8-10 pg/hr/10^6 HAECs) and RANTES (12-20 pg/hr/10^6 HAECs). Concentrations of MCP-2, Eotaxin, RANTES and IP-10 did not alter significantly upon treatment with TNFα or AICAR (data not shown).

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FIGURE LEGENDS:

Figure 1. AMPK-mediated inhibition of U-937 cell adhesion to HAECs.

HAECs were infected with the adenoviruses indicated for 24h prior to incubation in the presence or absence of TNFα (10ng/ml, 6h). Cells were co-incubated in the presence or absence of L-NAME (1mM, 6h) and AICAR (2mM, for the durations shown) prior to U-937 cell adhesion analysis. The results shown are from A) ten B) seven or C) twelve independent experiments performed in triplicate. * $p<0.01$ relative to value in absence of AICAR, $ $p<0.01$ relative to value in absence of L-NAME. ** $p<0.01$ relative to value in the presence of control adenoviruses.

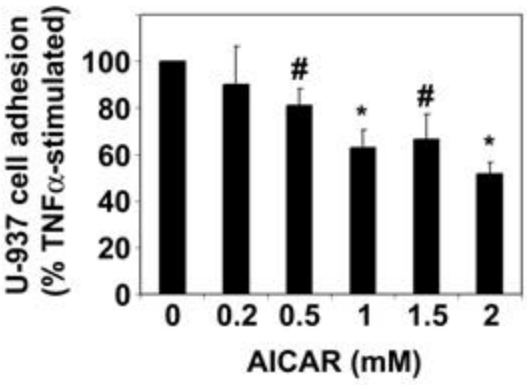
Figure 2. Effect of AMPK activation on adhesion molecule expression and MCP-1 secretion.

HAECs infected with the adenoviruses indicated were incubated in the presence or absence of TNFα (10ng/ml for 6h (A,B) or 4h (C,D)) and in the presence or absence of 2mM AICAR for the final 45 min (A,C,D) or 240 min (A,B). A,B) Cell surface expression of ICAM-1, VCAM-1 and E-selectin was assessed and the results are shown from nine independent experiments performed in triplicate. * $p<0.01$ relative to TNFα-stimulated value in absence of AICAR $ $p<0.01$ relative to value for HAECS infected with Ad.control C,D) Secreted MCP-1 was assessed from three independent experiments. **$p<0.05$ relative to value in absence of TNFα. #$p<0.05$ relative to value in the presence of Ad.control or L-NAME.

SUPPLEMENTAL FIGURES:

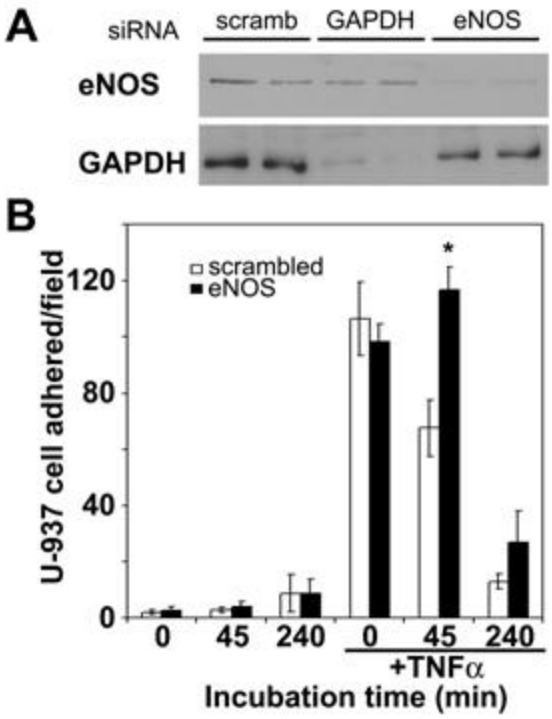
Supplemental Figure I. AICAR inhibits U-937 cell adhesion to HAECs in a dose-dependent manner.

TNFα-stimulated HAECs were co-incubated with the indicated concentrations of AICAR for the final 45min prior to U-937 cell adhesion analysis. The results shown are from ten independent experiments performed in triplicate. # $p<0.05$ relative to value in absence of AICAR. * $p<0.01$ relative to value in absence of AICAR.



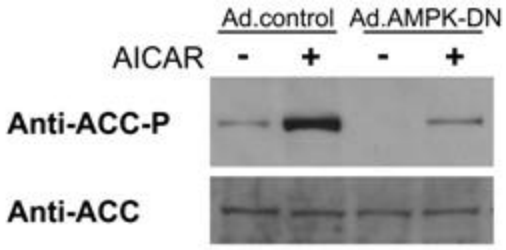
Supplemental Figure II. The effects of rapid, but not prolonged stimulation of AMPK on U-937 cell adhesion are eNOS-dependent.

HAECs were transfected with the siRNA indicated 48h prior to experimentation. A) Lysates were prepared from HAECs treated with the siRNA indicated and subjected to western blotting with the antibodies indicated. A representative immunoblot is shown. B) HAECs were incubated in the presence or absence of TNFα (6h, 10ng/ml) and 2mM AICAR for the final 45 or 240min. The results of seven independent adhesion assays are shown. * p<0.01 relative to value in the presence of scrambled siRNA.



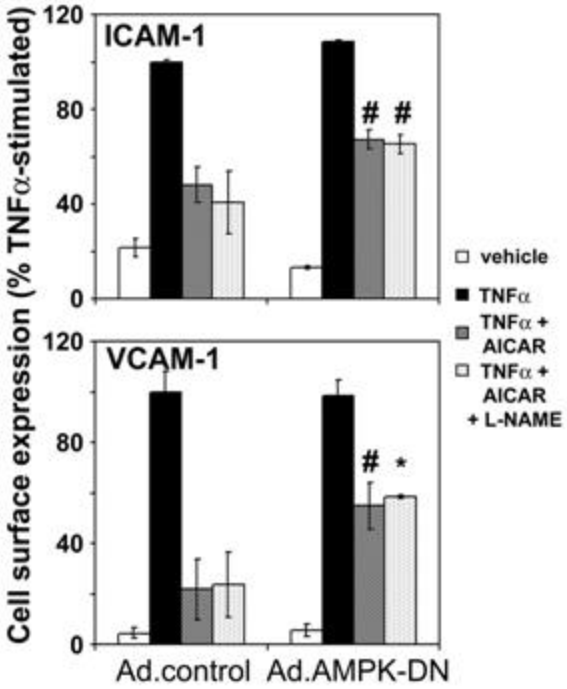
Supplemental Figure III. Inhibition of AICAR-stimulated AMPK activity by Ad.AMPK-DN.

Lysates were prepared from HAECs infected with the adenoviruses indicated after incubation in the presence or absence of 2mM AICAR for 45min and subjected to western blotting using anti-ACC and anti-phospho-ACC antibodies. Representative immunoblots are shown.



<u>Supplemental Figure IV.</u> The effects of L-NAME and Ad.AMPK-DN on AICAR-mediated inhibition of TNFa-stimulated ICAM-1 and VCAM-1 cell surface expression.

HAECs were infected with the adenoviruses indicated 24h prior to incubation in the presence or absence of TNFα (10 ng/ml) and L-NAME (1mM) for 6h. For the final 240min some cells were also incubated with 2 mM AICAR. HAEC surface expression of ICAM-1 and VCAM-1 was assessed and the results of nine independent experiments illustrated. *p<0.01 relative to value for HAECs infected with control viruses. #p<0.05 relative to value for HAECs infected with control viruses.



<u>Supplemental Figure V.</u> Rapid AMPK activation in HAECs reduces TNFa-stimulated U-937 cell chemotaxis in an AMPK and L-NAME-sensitive manner.

HAECs were infected with the adenoviruses indicated 24h prior to incubation in the presence or absence of 10ng/ml TNFα and/or 1mM L-NAME for 6h. For the final 45min some cells were also incubated with 2mM AICAR. HAECs were washed thoroughly and conditioned medium collected over 1h. U-937 cell migration towards conditioned medium was assessed and the results from three independent experiments are shown. *p<0.05 relative to value in the absence of AICAR. #p<0.05 relative to value in the presence of Ad.control. $p<0.05 relative to value in the absence of L-NAME.