

Nitric Oxide-Induced Activation of the AMP-Activated Protein Kinase $\alpha 2$ Subunit Attenuates I κ B Kinase Activity and Inflammatory Responses in Endothelial Cells

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Abstract

Background: In endothelial cells, activation of the AMP-activated protein kinase (AMPK) has been linked with anti-inflammatory actions but the events downstream of kinase activation are not well understood. Here, we addressed the effects of AMPK activation/deletion on the activation of NF κ B and determined whether the AMPK could contribute to the anti-inflammatory actions of nitric oxide (NO).

Methodology/Principal Findings: Overexpression of a dominant negative AMPK $\alpha 2$ mutant in tumor necrosis factor- α -stimulated human endothelial cells resulted in increased NF κ B activity, E-selectin expression and monocyte adhesion. In endothelial cells from AMPK $\alpha 2^{-/-}$ mice the interleukin (IL)-1 β induced expression of E-selectin was significantly increased. DETA-NO activated the AMPK and attenuated NF κ B activation/E-selectin expression, effects not observed in human endothelial cells in the presence of the dominant negative AMPK, or in endothelial cells from AMPK $\alpha 2^{-/-}$ mice. Mechanistically, overexpression of constitutively active AMPK decreased the phosphorylation of I κ B and p65, indicating a link between AMPK and the I κ B kinase (IKK). Indeed, IKK (more specifically residues Ser177 and Ser181) was found to be a direct substrate of AMPK $\alpha 2$ in vitro. The hyper-phosphorylation of the IKK, which is known to result in its inhibition, was also apparent in endothelial cells from AMPK $\alpha 2^{+/+}$ versus AMPK $\alpha 2^{-/-}$ mice.

Conclusions: These results demonstrate that the IKK is a direct substrate of AMPK $\alpha 2$ and that its phosphorylation on Ser177 and Ser181 results in the inhibition of the kinase and decreased NF κ B activation. Moreover, as NO potentially activates AMPK in endothelial cells, a portion of the anti-inflammatory effects of NO are mediated by AMPK.

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Introduction

The AMP-activated protein kinase (AMPK) is a member of the Snf1/AMPK family of serine/threonine protein kinases and is an evolutionarily conserved sensor of the cellular energy status. Although the AMPK pathway is traditionally thought of as an intracellular fuel gauge and regulator of metabolism, recent evidence indicates that it may also be important for the maintenance of endothelial function and to redress the disturbed redox balance associated with vascular disease. Certainly, the AMPK can influence a number of signaling cascades that would be expected to result in anti-atherosclerotic effects, such as attenuated free radical generation and the activation of angiogenic factors (for review see [1]).

Although the link between cellular metabolism and AMPK activation has been repeatedly demonstrated in tissues such as skeletal and cardiac muscle [2], the precise role played by the AMPK in endothelial cell remains incompletely understood. Indeed, while there are some situations in which activation of

the AMPK is reported to depend on an increase in the ADP/ATP ratio e.g. following cell stimulation with rosiglitazone [3], the activation of AMPK by Ca²⁺-elevating agonists such as bradykinin [4,5] and thrombin [6] has been attributed to the activity of an upstream activating kinase rather than to changes in AMP levels. There are two different isoforms of the catalytic α AMPK subunit ($\alpha 1$ and $\alpha 2$) that are differentially expressed in different tissues. For example, while the $\alpha 1$ isoform predominates in adipose tissue, skeletal muscle and cardiomyocytes express higher amounts of the AMPK $\alpha 2$ [7]. Interestingly, endothelial cells express both α subunits and different groups report the predominance of different isoforms, a finding that may explain the inconsistent dependence on changes in ADP/ATP for stimulation.

We reported previously that the AMPK can be activated by fluid shear stress as well as by NO in endothelial cells, and that it can affect the expression of endothelial cell proteins including, the hydroxy-methylglutaryl coenzyme A reductase, cytochrome P450 2C8, and angiotensin II [8–11]. Also the overexpression of dominant negative AMPK $\alpha 2$ in endothelial cells increases basal

and tumor necrosis factor (TNF)-α-stimulated E-selectin expression [10]. While the latter findings imply the involvement of the transcription factor nuclear factor κB (NFκB) and there are reports of an attenuated NFκB activation following AMPK activation in different cell types [12–15], the molecular mechanisms involved are not clear. Therefore, the aim of the present study was to address the link between AMPK activation and NFκB inhibition as well as to determine whether or not the activation of the AMPK could at least partially account for the effects of NO on NFκB activity and thus adhesion molecule expression.

Results

Effect of NO on the activation of AMPK and NFκB

Treatment of primary cultures of human endothelial cells with the NO donor DETA-NO (100 μmol/L) which has a t_{1/2} of 16 hours, elicited the time-dependent phosphorylation of the AMPK on Thr172 (Figure 1A). The phosphorylation of AMPK by exogenous NO was independent of the donor used as a substance with a markedly faster NO releasing kinetic i.e., DEA-NO, t_{1/2} 2 minutes, resulted in the more rapid activation of the AMPK i.e., within 2 minutes (Figure S1A). The effects were also concentration-dependent as indicated using a third NO donor with a more delayed NO release (DPTA NO, t_{1/2} 5 hours; Figure S1B). TNF-α (1 and 10 ng/ml, 30 min) elicited a marked and concentration-dependent increase in NFκB activity (EMSA; Figure 1B), that was attenuated by endothelial cell pre-treatment with DETA-NO (Figure 1B). TNF-α had however no acute effect on the activation of the AMPK in the absence of NO (Figure 1C).

Effect of constitutively active and dominant negative AMPK mutant on NFκB activation

To determine the involvement of the AMPK in the prevention of NFκB activation, we assessed the effects of constitutively active and dominant negative AMPKα2 mutants on the TNF-α-induced activation of NFκB. While TNF-α enhanced the activity of NFκB in cells infected with a control virus, this response was blunted in cells overexpressing the constitutively active AMPK (Figure 2A) and potentiated in cells expressing the dominant negative AMPK mutant (Figure 2B). To estimate the influence of the AMPK in the NO-mediated inhibition of TNF-α-induced NFκB activation, cells overexpressing either the control virus or the dominant negative AMPK mutant were exposed to TNF-α in the absence and presence of DETA-NO. As before, pre-treatment with the NO donor attenuated the activation of NFκB (Figure 2C). The effect of NO was however blunted in cells overexpressing the dominant negative AMPK mutant.

Effect of AMPK deletion on E-selectin and VCAM-1 expression in murine endothelial cells

Theoretically a dominant negative AMPKα2 mutant could affect the activity of the AMPKα1 by, for example, sequestering AMPKβ and γ subunits from the endogenous α1 subunit. To ensure that the effects observed could indeed be attributed to the AMPKα2, we performed additional experiments in endothelial cells from AMPKα2^{-/-} mice or their wild-type littermates (AMPKα2^{+/+}).

First, to determine whether or not the loss of one AMPKα subunit could result in the compensatory up regulation of the other we assessed AMPKα1 expression in aortae from α2^{+/+} and α2^{-/-} mice as well as AMPKα2 expression in α1^{+/+} and α1^{-/-} mice. We found that the deletion of the AMPKα2 did not affect α1

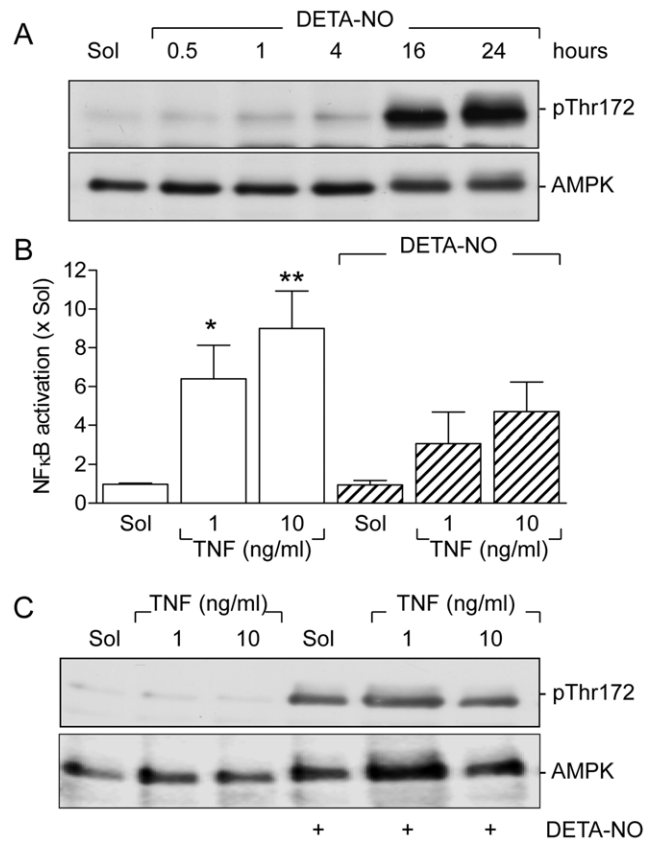


Figure 1. Effect of DETA-NO on AMPK and NFκB activation. Human endothelial cells were treated with either solvent (PBS) or DETA-NO (100 μmol/L) for (A) the times indicated or (C) 16 hours before stimulation with TNF-α (1 or 10 ng/ml) for 30 minutes. (A&C) Western blots showing the consequences of DETA-NO on the phosphorylation of the AMPK (p-Thr172) in the absence (A) and presence (C) of TNF-α. (B) The TNF-α-induced activation of NFκB in the absence and presence of DETA-NO was assessed by EMSA. The bar graph summarizes the results from 4–8 independent experiments; *P<0.05, **P<0.01 versus the appropriate Sol-treated group. The Western blots are representative of 2 additional experiments. doi:10.1371/journal.pone.0020848.g001

expression while the α2 subunit was upregulated in tissue from AMPKα1^{-/-} mice (Figure S2).

To determine the effect of AMPK deletion on the response to inflammatory mediators, murine microvascular endothelial cells from AMPKα2^{+/+} or AMPKα2^{-/-} mice were exposed to IL-1β (10 ng/mL, 6 hours) or to LPS (10 ng/mL, 6 hours) in the absence and presence of DETA-NO and the surface expression of E-selectin was assessed. The basal expression of E-selectin was slightly (but not significantly) enhanced in cells isolated from the AMPKα2^{-/-} compared to the AMPKα2^{+/+} animals. While the basal expression of the adhesion molecule was attenuated by NO, levels remained slightly elevated in the AMPKα2^{-/-} cells. Cell stimulation with IL-1β (Figure 3A) or LPS (Figure 3B) increased the expression of E-selectin with significantly higher expression levels being detected in AMPKα2^{-/-} cells. TNF-α failed to result in the activation of NFκB in either AMPKα2^{+/+} or AMPKα2^{-/-} cells studied. Pre-treatment of endothelial cells with the NO donor attenuated adhesion molecule expression but again expression was higher (2.5±1.0-fold for IL-1β and 1.7±0.4-fold for LPS) in the cells from AMPKα2^{-/-} mice than in cells from their wild-type littermates.

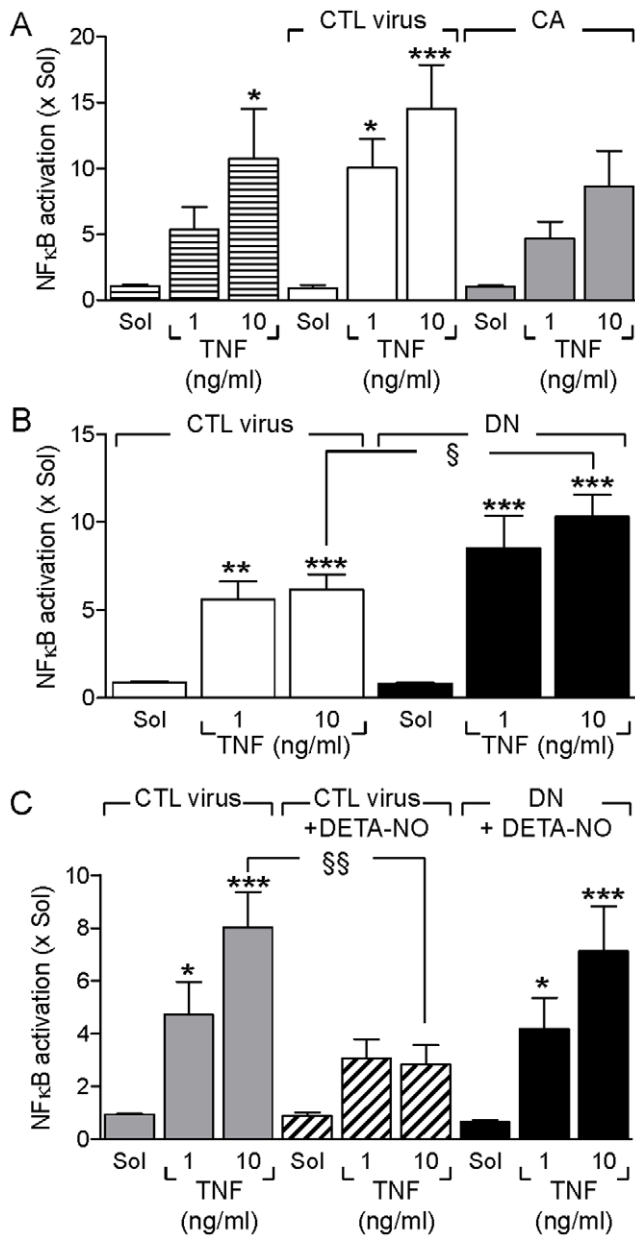


Figure 2. Effect of constitutively active and dominant negative AMPK mutants on NFκB activation. Human endothelial cells infected with either control (CTL) virus or adenoviral constructs encoding (A) constitutively active (CA) or (B&C) dominant negative (DN) AMPK mutants. After 48 hours cells were treated with TNF-α (1 or 10 ng/ml) for 30 minutes and NFκB activity determined by EMSA. In some experiments (C), cells were pre-treated with DETA-NO (100 μmol/L) prior to TNF-α stimulation. The bar graph summarizes the results of data obtained in 4 (A), 6 (B) or 5 (C) independent experiments; *P<0.05, **P<0.01, ***P<0.001 versus the appropriate Sol group and §P<0.05, §§P<0.01, §§§P<0.001 versus cells treated with CTL virus. doi:10.1371/journal.pone.0020848.g002

Effect of constitutively active and dominant negative AMPK mutants on cell adhesion

Next, we studied the consequences of AMPK mutant overexpression on the adhesion of mononuclear cells to endothelial cells. Consistent with the results obtained using the AMPKα2^{-/-} murine endothelial cells, significantly more mononuclear cells attached firmly to human endothelial cells overexpressing the dominant

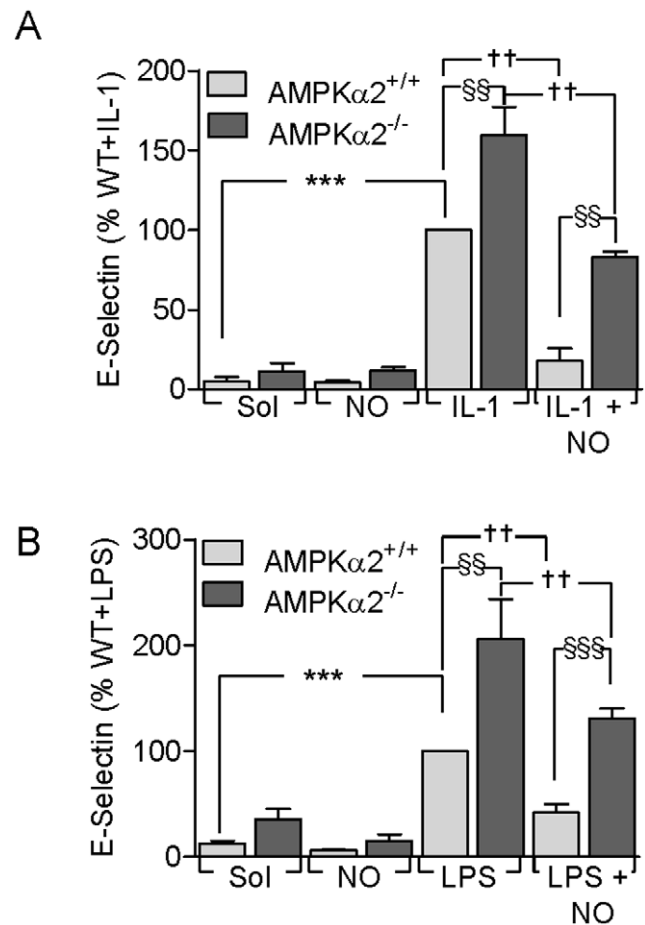


Figure 3. Effect of AMPK deletion on adhesion molecule expression and IκB stability. Lung endothelial cells from AMPKα2^{-/-} mice or their wild-type (+/+) littermates were treated with either IL-1β (10 ng/ml) or LPS (10 ng/ml) in the absence (or presence of DETA-NO (100 μmol/L, 24 hours) for 6 hours. Surface protein expression of (A) E-selectin and (B) VCAM-1 were determined by FACS analysis. The bar graphs summarize the results of data obtained in 4 independent experiments; ***P<0.001 versus Sol-treated cells, §§P<0.01, §§§P<0.001 and ††P<0.01. doi:10.1371/journal.pone.0020848.g003

negative AMPKα2 mutant than those expressing the constitutively active AMPK or treated with the control virus. Cell stimulation with TNF-α (1 ng/mL, 6 hours) resulted in a significant increase in mononuclear cell attachment (Figure 4) that was potentiated in cells expressing the dominant negative, but attenuated in cells expressing the constitutively active AMPK mutants.

Consequences of AMPK activation/inactivation on IκB

The activation of NFκB mainly occurs via the phosphorylation and degradation of inhibitory molecules, including IκB. Interestingly, the activation of the AMPK was associated with the increased expression of IκB protein, a phenomenon that was evident on comparing pulmonary endothelial cells from AMPKα2^{+/+} and AMPKα2^{-/-} mice (Figure 5A) or COS-7 cells overexpressing the constitutively active or dominant negative AMPKα2 mutants (Figure 5B). Both these findings support the above evidence indicating that activation of the AMPK results in NFκB inhibition. No difference in IκB expression was detected between AMPKα1^{+/+} and AMPKα1^{-/-} endothelial cells (data not shown).

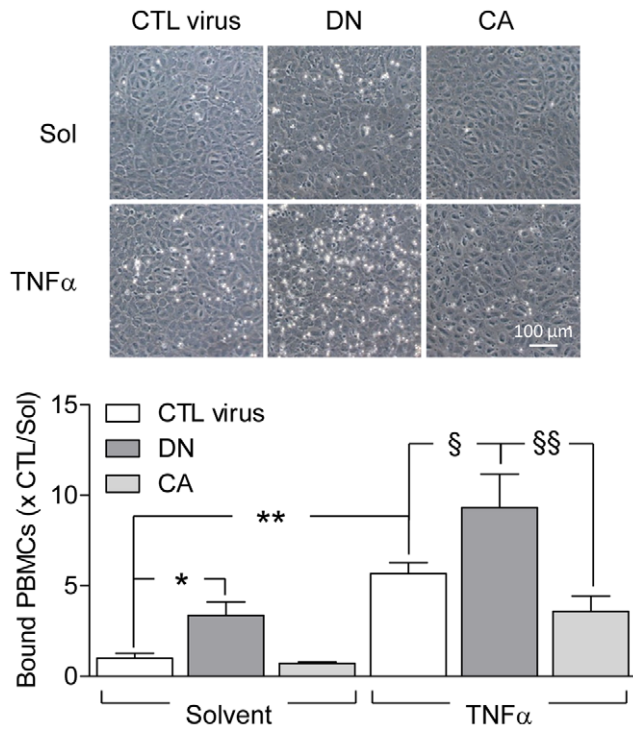


Figure 4. Effect of constitutively active and dominant negative AMPK mutants on monocyte adhesion. Human endothelial cells were infected with either a control (CTL) virus or adenoviral constructs encoding a constitutive active or dominant negative (DN) AMPK α 2 mutants. After 48 hours in culture, cells were treated with either solvent or TNF- α (10 ng/ml, 6 hours) before the addition of freshly isolated PBMCs (4×10^5 per well) for 10 minutes. The bar graph summarizes data obtained in 4 independent experiments, each performed in quadruplicate; * $P < 0.05$, ** $P < 0.01$ solvent treated cells expressing CTL virus and $\S P < 0.05$, $\S\S P < 0.01$ versus cells expressing the DN mutant. doi:10.1371/journal.pone.0020848.g004

COS-7 cells expressing constitutively active or dominant negative AMPK α 2 were used to determine the consequences of AMPK activation and inhibition on the I κ B kinase (IKK)-mediated phosphorylation of I κ B independent of potential interference by endogenously generated NO. Neither of the AMPK α 2 mutants studied affected the basal I κ B phosphorylation. However, following stimulation with TNF- α (10 ng/mL), I κ B phosphorylation was enhanced in cells expressing the dominant negative mutant (Figure 5C). The lack of effect of the constitutively active AMPK mutant in these cells can most probably be attributed to the high expression level and activity of the endogenous AMPK α isoforms in this cell line.

The TNF- α -activated IKK complex also phosphorylates p65 on Ser536, a step thought to be required for enhanced p65 transactivation potential and the optimal induction of NF κ B target genes [16]. The AMPK also seems to play a role in this pathway as we observed that the expression of the constitutively active AMPK led to a decrease in the TNF- α -stimulated phosphorylation of p65 (Figure S3). Similar results were obtained using human endothelial over expressing the AMPK α 2 but not the AMPK α 1 subunit (data not shown).

Consequences of AMPK activation/inactivation on IKK

Given that AMPK activation attenuated the phosphorylation of I κ B as well as p65 we proposed that the AMPK is able to attenuate NF κ B activation by phosphorylating and inhibiting the IKK. In *in*

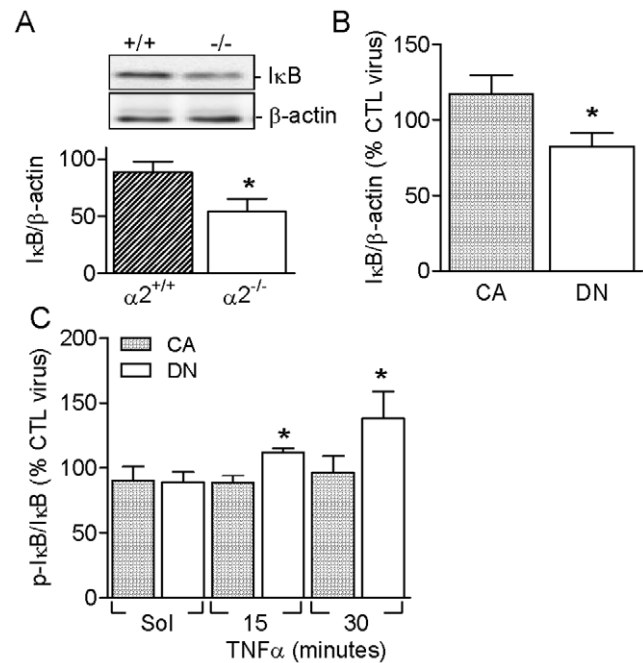


Figure 5. Role of AMPK α 2 in regulating the expression and phosphorylation of I κ B. Expression of I κ B in (A) pulmonary endothelial cells from AMPK α 2 $^{+/+}$ and AMPK α 2 $^{-/-}$ mice, and (B) in COS-7 cells expressing either constitutively active (CA) or dominant negative (DN) AMPK α 2. (C) I κ B phosphorylation in CA- or DN-AMPK α 2 expressing COS-7 cells and stimulated with solvent (Sol) or TNF- α (10 ng/mL). Data are expressed relative to values obtained in control virus-infected cells. The bar graphs summarize the results of 4 to 5 independent experiments; * $P < 0.05$ versus CA or $^{+/+}$. doi:10.1371/journal.pone.0020848.g005

in vitro kinase assays using the purified AMPK α 2 (together with β 1 and γ 1) we identified IKK β as an AMPK substrate (Figure 6A).

To determine the site(s) in IKK β phosphorylated by AMPK we replaced its major phosphorylation sites i.e. Ser177 and Ser181 with alanine by site-directed mutagenesis, and overexpressed these mutants in COS-7 cells. The wild-type and mutant IKK β were then immunoprecipitated and incubated with AMPK α 2. We found that the mutation of IKK β on Ser177 and Ser181 resulted in a lower level of phosphorylation than the wild-type enzyme. Moreover, phosphorylation was barely detectable in the S177A/S181A double mutant. When IKK β was replaced with GFP, no phosphorylation was observed in the presence of AMPK α 2 (Figure 6B). Also in COS-7 cells overexpressing a constitutively active AMPK α 2 mutant the phosphorylation of IKK (the antibody used recognizes phospho Ser177 and 181) was increased compared to that detected in cells treated with a control virus (Figure 6C). A similar, approximately 2-fold increase in pIKK levels was observed in murine endothelial cells stimulated with IL-1 β (30 ng/mL, Figure 6D) in that the phosphorylation of IKK was consistently greater in cells from AMPK α 2 $^{+/+}$ versus α 2 $^{-/-}$ mice. Signals from solvent-treated cells were too low to quantify. There was no difference in the IL-1 β -induced phosphorylation in AMPK α 1 $^{+/+}$ and AMPK α 1 $^{-/-}$ mice (Figure S4).

To assess IKK β activity we next determined the phosphorylation of GST-I κ B α in the presence of wild-type IKK β and either AMPK α 1 or α 2. We found that the phosphorylation of I κ B α was reduced in the presence of AMPK α 2 but not AMPK α 1 (Figure 6E).

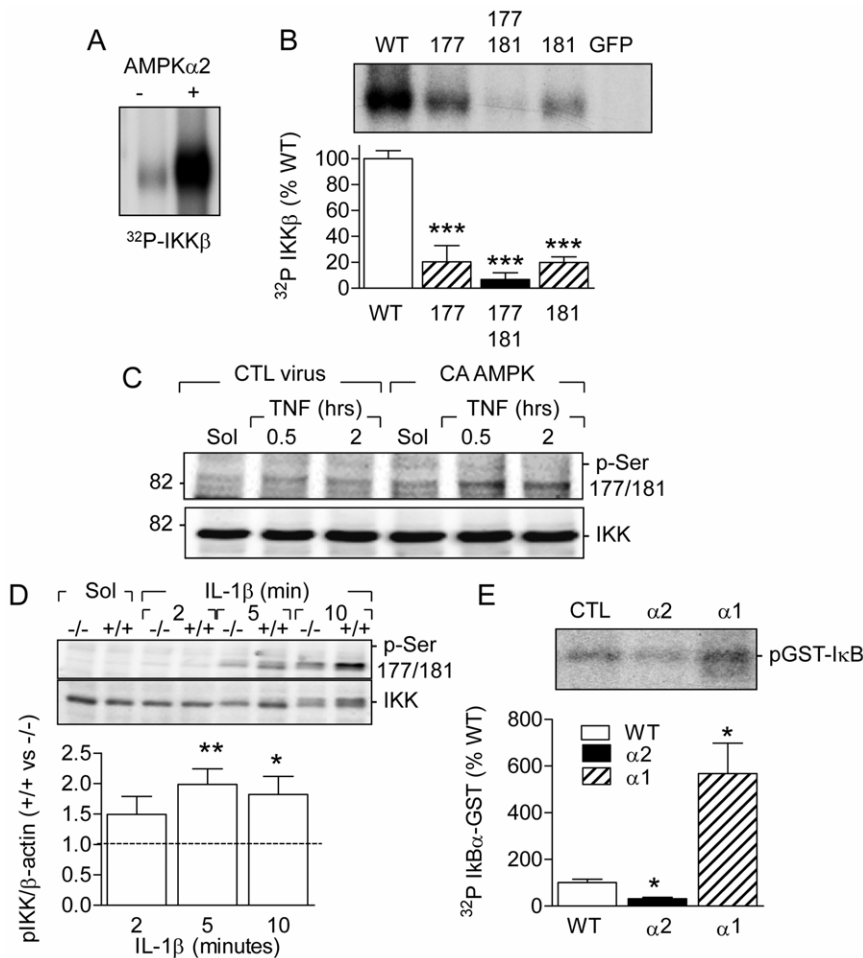


Figure 6. Identification of IKK as an AMPK α 2 substrate. (A) Autoradiograph showing the in vitro phosphorylation (32 P) of purified IKK β by AMPK α 2. Similar results were obtained in 2 additional experiments. (B) Effect of the substitution of Ser177 and Ser181 with alanine on the AMPK α 2-dependent phosphorylation of IKK β . GFP was used instead of IKK β as a negative control. (C) Phosphorylation of IKK in solvent (Sol)- or TNF- α -stimulated COS-7 cells 48 hours after infection with either CTL or constitutively active (CA) AMPK α 2 adenoviruses. Similar results were obtained in 2 additional experiments. (D) Phosphorylation of IKK in solvent (Sol)- or IL-1 β -stimulated endothelial cells from AMPK α 2 $^{+/+}$ and α 2 $^{-/-}$ mice. (E) Autoradiograph of phosphorylated GST-I κ B after IKK activity assay in the presence of protein A/G (CTL) or immunoprecipitated AMPK α 2 or AMPK α 1 subunits. Similar results were obtained in three different experiments. The bar graphs summarize the data from 4 (B and E) to 8 (D) independent experiments; *P<0.05, **P<0.01 ***P<0.001 versus WT or $^{+/+}$. doi:10.1371/journal.pone.0020848.g006

Discussion

The results of the present investigation indicate that the AMPK α 2 subunit plays an important role in regulating inflammatory responses, adhesion molecule expression (E-selectin and VCAM-1) and monocyte adherence to endothelial cells. These effects could be related, at least partly to the AMPK-mediated phosphorylation of IKK and subsequent inhibition of I κ B and p53 phosphorylation as well as DNA binding (see Figure 7). Moreover, three different NO donors were able to activate the AMPK and the NO-mediated inhibition of NF κ B activation was attenuated by a dominant negative AMPK in human endothelial cells as well as in endothelial cells from AMPK α 2 $^{-/-}$ mice. Thus, it appears that the NO-mediated inhibition of NF κ B activity is, at least partially, dependent on the activation of the AMPK α 2 subunit.

The possibility that the AMPK can regulate the DNA binding activity of NF κ B was initially indicated by the fact that overexpression of a dominant negative AMPK increased, while a constitutively active AMPK decreased the TNF- α -induced expression of E-selectin [10]. There have since been reports linking

AMPK with NF κ B in endothelial cells, but the overall outcome of kinase activation is controversial as both NF κ B inhibition [12,17–19] and activation [20–23] have been reported. Whether or not this discrepancy can be attributed to the parallel activation/inactivation of associated regulatory mechanisms and signal

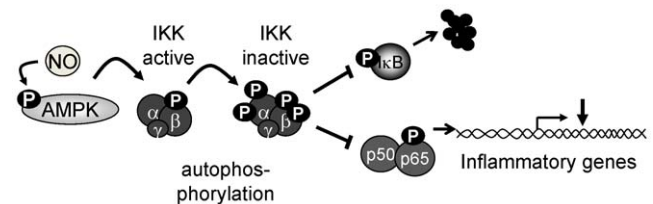


Figure 7. Scheme of the mechanism proposed. NO Initiates the activation of the AMPK α 2 in endothelial cells which in turn phosphorylates and activates the β -subunit of the IKK. The latter also induces a higher rate of IKK auto-inactivation and thus attenuates the activation of NF κ B and the expression of inflammatory genes. doi:10.1371/journal.pone.0020848.g007

transduction pathways, remains to be determined. One important consideration is that much of the published data was generated using the AMPK activator, 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR), which can elicit AMPK-independent effects [24], and substances such as compound C and iodotubercidin which are by no means specific inhibitors of the AMPK. Therefore, one focus of the current investigation was to make use of the available constitutively active and dominant negative AMPK mutants and endothelial cells from AMPK $\alpha 2^{+/+}$ and AMPK $\alpha 2^{-/-}$ mice to determine which of the catalytic subunits was most likely to affect transcription factor activity. The results obtained using murine lung endothelial cells indicate that the AMPK $\alpha 2$ plays a prominent role in modulating NF κ B phosphorylation in endothelial cells.

Which point in the NF κ B activation cascade could be affected by AMPK? NF κ B is a dimer consisting of the transcription factors p65 (RelA) or p50, and under resting conditions it is associated with its inhibitory protein I κ B which retains the complex in the cytosol. The activation of NF κ B mainly occurs via the phosphorylation of I κ B, which results in its degradation - leaving the NF κ B dimer free to translocate into the nucleus and stimulate transcription [16]. Using dominant negative and constitutively active AMPK mutants we found that the phosphorylation of I κ B (residue Ser32 or Ser36) was increased when the AMPK was inhibited but decreased when AMPK activity was increased. We also observed the attenuated basal expression of I κ B in the AMPK $\alpha 2^{-/-}$ versus AMPK $\alpha 2^{+/+}$ endothelial cells. As the expression of I κ B is known to be regulated by the activity of NF κ B [25], these data further highlight the importance of the AMPK $\alpha 2$ subunit in the regulation of transcription factor activity.

The serine phosphorylation of I κ B is mediated by a large multi-unit complex containing two catalytic subunits (IKK α and IKK β) as well as the regulatory subunit IKK γ or NEMO, which has no kinase domain. The p65 subunit is also a IKK substrate and p65 phosphorylation on Ser536 is thought to be required for enhanced p65 transactivation potential and the optimal induction of NF κ B target genes [16]. Given that we observed that both AMPK activation and a constitutively active AMPK mutant decreased the phosphorylation of I κ B and p65, it seemed logical to conclude that the AMPK is able to modulate the activity of the IKK complex.

To date there has only been circumstantial evidence to suggest a role of the AMPK in the modulation of IKK activity. For example, AICAR has been reported to be without effect on IKK in rat skeletal muscle cells [26] but to attenuate I κ B phosphorylation in IL-18-stimulated cardiac microvascular endothelial cells [27]. Moreover, the overexpression of a dominant-negative AMPK in TNF- α -stimulated mouse macrophages resulted in the accelerated and exaggerated degradation of I κ B [28]. Similarly, during the preparation of this manuscript the loss of AMPK activity was reported to result in increased I κ B α degradation in cultured endothelial cells [23], a finding we have confirmed in native endothelial cells. The results of the present study clearly indicate that the AMPK can directly phosphorylate and attenuate the activity of the IKK in native and cultured endothelial cells. In *in vitro* assays we identified IKK β as an AMPK substrate and were able to detect the elevated phosphorylation of IKK in intact AMPK-overexpressing cells. The phosphorylation of IKK β on Ser 177 and/or 181 causes the rapid phosphorylation of a C-terminal serine cluster which in turn elicits the auto-inhibition of kinase activity and is thought to be an effective means of limiting the duration of IKK activation [29]. Our data suggest that the AMPK $\alpha 2$ is able to phosphorylate both of these serine residues as mutation of a single site reduced phosphorylation which was only barely detectable in a double S177A/S181A mutant. That the AMPK-mediated phos-

phorylation of the IKK most likely results in NF κ B inhibition was demonstrated by the fact that the IKK-dependent phosphorylation of a I κ B-GST construct was markedly attenuated in the presence of the AMPK $\alpha 2$ but not the AMPK $\alpha 1$ subunit. Moreover, the expression of I κ B was significantly higher in human endothelial cells overexpressing the constitutively active AMPK $\alpha 2$ and in AMPK $\alpha 2^{+/+}$ versus AMPK $\alpha 2^{-/-}$ mouse endothelial cells.

The AMPK has been implicated in the phosphorylation and activation of the endothelial NO synthase (eNOS), at least *in vitro*, and thus one further mechanism by which the AMPK could affect NF κ B would be by modulating basal NO output (reviewed in [1]); which is known to decrease NF κ B activity [30]. However, using COS-7 cells which do not express endogenous eNOS, we found that AMPK activation and the constitutively active AMPK were sufficient to attenuate the TNF- α -induced phosphorylation of I κ B and p65. Thus in our hands, the AMPK-dependent inhibition of NF κ B was not dependent on NO production. In fact, we were able to confirm that NO donors are effective activators of the AMPK a finding that fits well with previous reports in endothelial cells that AMPK activation is markedly attenuated in the presence of a NOS inhibitor or in cells from eNOS $^{-/-}$ mice [5,9]. How NO activates the AMPK remains to be elucidated but may well be related to the activation of the Ca $^{2+}$ /calmodulin-dependent protein kinase kinase β , a well known AMPK kinase [5].

Taken together, the results of the present investigation indicate that the activation of the AMPK in endothelial cells can limit inflammatory responses via the phosphorylation and inhibition of IKK activity. Moreover, as the AMPK could be activated by NO, the AMPK dependent inhibition of IKK activity may contribute to the anti-inflammatory and anti-atherosclerotic actions of the endothelium-derived autacoid.

Materials and Methods

Materials

Antibodies for Western blotting directed against phospho-Thr172 AMPK and total AMPK, phospho-Ser536 NF κ B, phospho-Ser32/36 I κ B and phospho Ser 177/181 I κ B kinase (IKK) were purchased from New England Biolabs (Frankfurt, Germany). Antibodies recognizing I κ B, NF κ B or IKK from Santa Cruz Biotechnology (Heidelberg, Germany). Horseradish peroxidase conjugated secondary antibodies were from Calbiochem (Merck, Darmstadt, Germany) and the fluorescent antibodies recognizing E-selectin and VCAM-1 used for FACS analyses were from BD Biosciences (Pharmingen, Germany). TNF- α and interleukin (IL)-1 β , were from PeproTech (Cell Concept, Umkirch, Germany), and DETA-NONOate (DETA-NO), DEA-NONOate and DPTA-NONOate were from Alexis (Lörrach, Germany). The antibody against β -actin and other chemicals were obtained from Sigma (Munich, Germany).

Cell culture

Human umbilical vein endothelial cells were isolated and cultured as described [31]. The use of human material in this study conforms to the principles outlined in the Declaration of Helsinki [32] and was confirmed in written form by the members of the Ethic-Commission of the Medical Faculty of the Goethe-University (Frankfurt am Main, Germany) and the donors gave verbal consent. Murine lung endothelial cells were isolated and cultured as described [8], from AMPK $\alpha 1^{-/-}$ or AMPK $\alpha 2^{-/-}$ mice or the respective littermate wild-type animals [33,34] (kindly provided by Benoit Viollet, Paris via the European Mouse Mutant Archive, Munich, Germany). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by

the European Commission Directive 86/609/EEC. Both the University Animal Care Committee and the Federal Authority for Animal Research at the Regierungspräsidium Darmstadt (Hessen, Germany) gave written approval to the study protocol (# F28/17). For the isolation of the pulmonary endothelial cells, mice were sacrificed using 4% isoflurane in air and subsequent exsanguination. COS-7 cells were purchased from the American Tissue Culture Collection (LCG Standards, Wesel, Germany) and cultured in Minimal Essential Medium (Invitrogen, Karlsruhe, Germany) supplemented with 8% fetal calf serum, pyruvate, non essential amino acids and gentamycin.

Adenoviral transduction of endothelial cells

Subconfluent endothelial cells (1st passage) were infected with adenoviruses (provided by Ken Walsh, Boston and Benoit Viollet, Paris, France) to over-express constitutively active AMPK α 2 [35], or dominant-negative AMPK α 2 [36], as described [10]. As the viral backbones of the constitutive active and dominant negative viruses were not identical, the observed effects were always analyzed to the respective control virus.

Immunoblotting

Protein samples were heated with SDS-PAGE sample buffer and separated by SDS-PAGE as described [8]. Proteins were detected using their respective antibodies and enhanced chemiluminescence using a commercially available kit (Amersham, Germany). To assess the phosphorylation of proteins, either equal amounts of protein from each sample were loaded twice and one membrane incubated with the phospho-specific antibody and the other with an antibody recognizing total protein, or blots were re-probed with the appropriate antibody.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear and cytosolic proteins were isolated, and binding to γ [³²P]-ATP (Hartmann Analytic, Braunschweig, Germany)-labeled double-stranded oligonucleotides containing the consensus sequence of the binding site for transcription factor NF κ B (5-AGT TGA GGG GAC TTT CCC AGG C-3, Santa Cruz) was assessed as described [37].

Flow cytometry

Following stimulation endothelial cells were harvested using accutase (PAA laboratories, Coelbe, Germany) and washed twice with PBS. After blocking (1% BSA, 15 minutes, 4°C) cells were incubated with the specific conjugated antibodies (30 minutes, 4°C), washed twice with PBS and fixed in 1% paraformaldehyde. Antibody binding was analyzed using a flow cytometer (FACSCalibur, BD) by counting 20,000 cells per sample. The values are presented following subtraction of the isotype-matched, control IgG.

Adhesion assay

Peripheral blood mononuclear cells were freshly isolated from blood obtained from healthy volunteers using a Biocoll[®] (1.077 g/ml) gradient (Biochrom AG, Berlin, Germany) according to the manufacturer's protocol. Confluent cultures of human endothelial cells were stimulated with TNF- α for 6 hours, and were washed with culture medium before freshly isolated mononuclear cells (400,000 per 12 well) were added. After 10 minutes of incubation the non-adherent cells were removed the number of firmly adherent cells was quantified.

IKK β kinase assays

The phosphorylation of IKK β by AMPK α 2 and the activity of IKK β were assessed in in vitro kinase assays. Ser177 and Ser181 in a flag-tagged IKK plasmid [38] (Addgene, Cambridge, MA) were mutated to alanine by site-directed mutagenesis (Quick exchange Kit, Agilent, Böblingen, Germany) using specific oligonucleotides (Biospring, Frankfurt, Germany). COS-7 cells were transfected with a plasmid encoding either the wild-type flag-tagged IKK β or one of the S177A, S181A or S177A/181A mutants, which were then immunoprecipitated using a Flag antibody (Invitrogen, Karlsruhe, Germany). The AMPK α 2 subunit was also immunoprecipitated from COS-7 cells and the in vitro kinase reaction performed as described [9]. In some experiments the activity of the IKK β was detected by monitoring the phosphorylation of a GST-I κ B fusion protein (kindly provided by Fumiyo Ikeda, Frankfurt).

Statistical analysis

Values are expressed as the mean \pm SEM and statistical evaluation was performed using Student's *t* test for unpaired data and one-way ANOVA or ANOVA for repeated measures followed by followed by a Bonferroni *t* test where appropriate. Values of *P*<0.05 were considered statistically significant.

Supporting Information

Figure S1 Effect of NO donors on the phosphorylation of AMPK. (A) Human endothelial cells (passage 2) were treated with either solvent (Sol) or DEA-NO (100 μ mol/L, *t*_{1/2} 16 minutes) for up to 30 minutes. Identical results were obtained in two additional experiments. (B) Human endothelial cells were treated with different concentrations of the NO donor DPTA NONOate (*t*_{1/2} 5 hours) and the phosphorylation of the AMPK was detected by Western blotting. The graph summarizes the data obtained in 3 independent experiments; ***P*<0.01 versus the appropriate Sol-treated group. (TIF)

Figure S2 Effect of AMPK α subunit deletion on the expression of the second isoform in aortic lysates. While deletion of the AMPK α 2 subunit had no effect on the expression of the AMPK α 1 isoform, the deletion of AMPK α 1 induced a compensatory increase in AMPK α 2 expression. Each lane represents tissue from a different animal and identical results were obtained in tissue from 4 additional animals. (TIF)

Figure S3 Role of AMPK α 2 in regulating the expression and phosphorylation of p65. The phosphorylation of p65 NF- κ B was assessed in COS-7 cells expressing either constitutively active (CA) or dominant negative (DN) AMPK α 2 and stimulated with solvent (Sol) or TNF- α (10 ng/mL). Data are expressed relative to values obtained in control virus-infected cells. The bar graph summarizes the results of 4 to 5 independent experiments; **P*<0.05 versus CA. (TIF)

Figure S4 Effect of the AMPK α 1 deletion on the IL1 β (30 ng/mL)-mediated phosphorylation of IKK α / β and I κ B in mouse lung endothelial cells. The bar graph summarizes the results of 4–5 independent experiments. (TIF)

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References

- Fisslthaler B, Fleming I (2009) Activation and signaling by the AMP-activated protein kinase in endothelial cells. *Circ Res* 105: 114–127.
- Arad M, Seidman CE, Seidman JG (2007) AMP-activated protein kinase in the heart: role during health and disease. *Circ Res* 100: 474–488.
- Boyle JG, Logan PJ, Ewart MA, Reihill JA, Ritchie SA, et al. (2008) Rosiglitazone stimulates nitric oxide synthesis in human aortic endothelial cells via AMP-activated protein kinase. *J Biol Chem* 283: 11210–11217.
- Mount PF, Lane N, Venkatesan S, Steinberg GR, Fraser SA, et al. (2008) Bradykinin stimulates endothelial cell fatty acid oxidation by CaMKK-dependent activation of AMPK. *Atherosclerosis* 200: 28–36.
- Zhang J, Xie Z, Dong Y, Wang S, Liu C, et al. (2008) Identification of nitric oxide as an endogenous activator of the AMP-activated protein kinase in vascular endothelial cells. *J Biol Chem* 283: 27452–27461.
- Stahmann N, Woods A, Carling D, Heller R (2006) Thrombin activates AMP-activated protein kinase in endothelial cells via a pathway involving Ca²⁺/calmodulin-dependent protein kinase kinase β. *Mol Cell Biol* 26: 5933–5945.
- Li J, Coven DL, Miller EJ, Hu X, Young ME, et al. (2006) Activation of AMPK α- and γ-isoform complexes in the intact ischemic rat heart. *Am J Physiol Heart Circ Physiol* 291: H1927–H1934.
- Fleming I, Fisslthaler B, Dixit M, Busse R (2005) Role of PECAM-1 in the shear-stress-induced activation of Akt and the endothelial nitric oxide synthase (eNOS) in endothelial cells. *J Cell Sci* 118: 4103–4111.
- Fisslthaler B, Fleming I, Keserü B, Walsh K, Busse R (2007) Fluid shear stress and NO decrease the activity of the hydroxy-methylglutaryl coenzyme A reductase in endothelial cells via the AMP-activated protein kinase and FoxO1. *Circ Res* 100: e12–e21.
- Dixit M, Bess E, Fisslthaler B, Hartel FV, Noll T, et al. (2008) Shear stress-induced activation of the AMP-activated protein kinase regulates FoxO1a and angiopoietin-2 in endothelial cells. *Cardiovasc Res* 77: 160–168.
- Webler AC, Michaelis UR, Popp R, Barbosa-Sicard E, Murugan A, et al. (2008) Epoxyeicosatrienoic acids are part of the VEGF-activated signaling cascade leading to angiogenesis. *Am J Physiol Cell Physiol* 295: C1292–C1301.
- Cacicedo JM, Yagihashi N, Keane JF Jr, Ruderman NB, Ido Y (2004) AMPK inhibits fatty acid-induced increases in NF-κB transactivation in cultured human umbilical vein endothelial cells. *Biochem Biophys Res Commun* 324: 1204–1209.
- Okayasu T, Tomizawa A, Suzuki K, Manaka K, Hattori Y (2008) PPARα activators upregulate eNOS activity and inhibit cytokine-induced NF-κB activation through AMP-activated protein kinase activation. *Life Sci* 82: 884–891.
- Suzuki K, Uchida K, Nakanishi N, Hattori Y (2008) Cilostazol activates AMP-activated protein kinase and restores endothelial function in diabetes. *Am J Hypertens* 21: 451–457.
- Hattori Y, Nakano Y, Hattori S, Tomizawa A, Inukai K, Kasai K (2008) High molecular weight adiponectin activates AMPK and suppresses cytokine-induced NF-κB activation in vascular endothelial cells. *FEBS Lett* 582: 1724.
- Viatour P, Merville MP, Bours V, Chariot A (2005) Phosphorylation of NF-κB and IκB proteins: implications in cancer and inflammation. *Trends Biochem Sci* 30: 43–52.
- Devaraj S, Torok N, Dasu MR, Samols D, Jialal I (2008) Adiponectin decreases C-reactive protein synthesis and secretion from endothelial cells: evidence for an adipose tissue-vascular loop. *Arterioscler Thromb Vasc Biol* 28: 1368–1374.
- Hattori Y, Suzuki K, Tomizawa A, Hiramata N, Okayasu T, et al. (2009) Cilostazol inhibits cytokine-induced nuclear factor-κB activation via AMP-activated protein kinase activation in vascular endothelial cells. *Cardiovasc Res* 81: 133–139.
- Wang C, Li L, Zhang ZG, Fan D, Zhu Y, et al. (2010) Globular adiponectin inhibits angiotensin II-induced nuclear factor kappaB activation through AMP-activated protein kinase in cardiac hypertrophy. *J Cell Physiol* 222: 149–155.
- Tomizawa A, Hattori Y, Kasai K (2009) Induction of gene expression in response to globular adiponectin in vascular endothelial cells. *Life Sci* 85: 457–461.
- Bair AM, Thippogowda PB, Freichel M, Cheng N, Ye RD, et al. (2009) Ca²⁺ entry via TRPC channels is necessary for thrombin-induced NF-κB activation in endothelial cells through AMP-activated protein kinase and protein kinase Cδ. *J Biol Chem* 284: 563–574.
- Liu C, Liang B, Wang Q, Wu J, Zou MH (2010) Activation of the AMP-activated protein kinase α1 alleviates endothelial cell apoptosis by increasing the expression of anti-apoptotic proteins BCL-2 and survivin. *J Biol Chem* 285: 15346–15355.
- Wang S, Zhang M, Liang B, Xu J, Xie Z, et al. (2010) AMPKα2 deletion causes aberrant expression and activation of NAD(P)H oxidase and consequent endothelial dysfunction in vivo. Role of 26S proteasomes. *Circ Res* 106: 1117–1128.
- Guigas B, Taleux N, Foretz M, Demaille D, Andreelli F, et al. (2007) AMP-activated protein kinase-independent inhibition of hepatic mitochondrial oxidative phosphorylation by AICA riboside. *Biochem J* 404: 499–507.
- Sun SC, Ganchi PA, Ballard DW, Greene WC (1993) NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregulatory pathway. *Science* 259: 1912–1915.
- Ho RC, Hirshman MF, Li Y, Cai D, Farmer JR, et al. (2005) Regulation of IκB kinase and NF-κB in contracting adult rat skeletal muscle. *Am J Physiol Cell Physiol* 289: C794–C801.
- Chandrasekar B, Boylston WH, Venkatachalam K, Webster NJG, Prabhu SD, et al. (2008) Adiponectin blocks interleukin-18-mediated endothelial cell death via APPL1-dependent AMP-activated protein kinase (AMPK) activation and IKK/NF-κB/PTEN suppression. *J Biol Chem* 283: 24889–24898.
- Sag D, Carling D, Stout RD, Suttles J (2008) Adenosine 5'-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. *J Immunol* 181: 8633–8641.
- Delhase M, Hayakawa M, Chen Y, Karin M (1999) Positive and negative regulation of IκB kinase activity through IKK subunit phosphorylation. *Science* 284: 309–313.
- Zeicher AM, Fisslthaler B, Schray-Utz B, Busse R (1995) Nitric oxide modulates the expression of monocyte chemoattractant protein 1 in cultured human endothelial cells. *Circ Res* 76: 980–986.
- Busse R, Lamontagne D (1991) Endothelium-derived bradykinin is responsible for the increase in calcium produced by angiotensin-converting enzyme inhibitors in human endothelial cells. *Naunyn-Schmiedeberg Arch Pharmacol* 344: 126–129.
- World Medical Association (1997) World Medical Association Declaration of Helsinki: Recommendations guiding physicians in biomedical research involving human subjects. *Cardiovascular Research* 35: 2–3.
- Jorgensen SB, Viollet B, Andreelli F, Frosig C, Birk JB, et al. (2004) Knockout of the α2 but not α1 5'-AMP-activated protein kinase isoform abolishes 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside but not contraction-induced glucose uptake in skeletal muscle. *J Biol Chem* 279: 1070–1079.
- Viollet B, Andreelli F, Jorgensen SB, Perrin C, Geloen A, et al. (2003) The AMP-activated protein kinase α2 catalytic subunit controls whole-body insulin sensitivity. *J Clin Invest* 111: 91–98.
- Woods A, Azzout-Marniche D, Foretz M, Stein SC, Lemarchand P, et al. (2000) Characterization of the role of AMP-activated protein kinase in the regulation of glucose-activated gene expression using constitutively active and dominant negative forms of the kinase. *Mol Cell Biol* 20: 6704–6711.
- Foretz M, Ancellin N, Andreelli F, Saintillan Y, Grondin P, et al. (2005) Short-term overexpression of a constitutively active form of AMP-activated protein kinase in the liver leads to mild hypoglycemia and fatty liver. *Diabetes* 54: 1331–1339. 54/5/1331 [pii].
- Schini-Kerth VB, Boese M, Busse R, Fisslthaler B, Mülsch A (1997) N-α-tosyl-L-lysine chloromethylketone prevents expression of iNOS in vascular smooth muscle by blocking activation of NF-κB. *Arterioscler Thromb Vasc Biol* 17: 672–679.
- Gelezunas R, Ferrell S, Lin X, Mu Y, Cunningham ET, Jr, et al. (1998) Human T-cell leukemia virus type 1 Tax induction of NF-κB involves activation of the IκB kinase α (IKKα) and IKKβ cellular kinases. *Mol Cell Biol* 18: 5157–5165.

Author Contributions

Conceived and designed the experiments: BF IF. Performed the experiments: EB BF TF. Analyzed the data: EB BF TF IF. Contributed reagents/materials/analysis tools: EB BF TF. Wrote the paper: IF.