Activation of AMPK stimulates heme oxygenase-1 gene expression and human endothelial cell survival

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Liu X, Peyton KJ, Shebib AR, Wang H, Korthuis RJ, Durante W. Activation of AMPK stimulates heme oxygenase-1 gene expression and human endothelial cell survival. Am J Physiol Heart Circ Physiol 300: H84–H93, 2011. First published October 29, 2010; doi:10.1152/ajpheart.00749.2010.—The present study determined whether AMP-activated protein kinase (AMPK) regulates heme oxygenase (HO)-1 gene expression in endothelial cells (ECs) and if HO-1 contributes to the biological actions of this kinase. Treatment of human ECs with the AMPK activator 5-aminimidazole-4-carboxamide-1-β-β-β-ribofuranoside (AICAR) stimulated a concentration- and time-dependent increase in HO-1 protein and mRNA expression that was associated with a prominent increase in nuclear factor-erythroid 2-related factor 2 (Nrf2) protein. Induction of HO-1 was also observed in rat carotid arteries after the in vivo application of AICAR. Induction of HO-1 by AICAR was blocked by the AMPK inhibitor compound C, the adenosine kinase inhibitor 5′-iodotubercidin, and by silencing AMPK-α1,2 and was mimicked by the AMPK activator A-769662 and by infecting ECs with an adenovirus expressing constitutively active AMPK-α1. AICAR also induced a significant rise in HO-1 promoter activity that was abolished by mutating the antioxidant responsive elements of the HO-1 promoter or by the overexpression of dominant negative Nrf2. Finally, activation of AMPK inhibited cytokine-mediated EC death, and this was prevented by the HO inhibitor tin protoporphyrin-IX or by silencing HO-1 expression. In conclusion, AMPK stimulates HO-1 gene expression in human ECs via the Nrf2/antioxidant responsive element signaling pathway. The induction of HO-1 mediates the antiapoptotic effects of AMPK, and this may provide an important adaptive response to preserve EC viability during periods of metabolic stress.

vascular biology; metabolic stress; endothelium; AMP-activated protein kinase

AMP-activated protein kinase (AMPK) is a ubiquitously expressed energy-sensing enzyme that functions as a protein serine/threonine kinase (17). AMPK exists as a heterotrimeric complex composed of α-, β-, and γ-subunits. The α-subunit of AMPK contains the catalytic domain and has two isoforms, α1 and α2, that are phosphorylated at Thr172 upon enzyme activation. Both α-isooforms of AMPK are expressed by endothelial cells (ECs); however, the predominant isoform is the α1-isoform (13, 43). In mammalian cells, AMPK is activated by increases in the AMP-to-ATP ratio, which occur in various stress conditions such as nutrient deprivation, prolonged exercise, hypoxia, ischemia, and heat shock (21). Binding of AMP to the α-subunit results in the partial allosteric activation of AMPK, and the enzyme becomes fully activated after the phosphorylation of Thr172 by AMPK kinases (13, 43). However, numerous physiological and pharmacologically relevant molecules are also capable of activating AMPK independent of changes in the AMP-to-ATP ratio (13, 14, 43). Once activated, AMPK coordinates a cellular program that prevents further ATP depletion by switching on catabolic pathways that generate ATP and turning off ATP-consuming anabolic pathways. AMPK mediates these effects through the direct phosphorylation of target proteins and by regulating gene expression (13, 17, 43).

A number of pharmacological activators of AMPK have been developed to probe AMPK function. 5-Aminimidazole-4-carboxamide-1-β-β-β-β-ribofuranoside (AICAR) is a well-established, cell-permeable activator of AMPK. Upon entering cells, AICAR is metabolized by adenosine kinase to 5-aminimidazole-4-carboxamide, which mimics the effect of AMP on AMPK activation (7). More recently, the thienopyridone compound A-769662 has been identified as a potent and highly selective activator of AMPK. This small molecule directly activates AMPK in a manner similar to that of AMP, encompassing allosteric activation as well as protection from Thr172 dephosphorylation (16).

Although AMPK has traditionally been viewed as a modulator of metabolism, recent studies have demonstrated that AMPK also functions to regulate endothelial function. AMPK phosphorylates and activates endothelial nitric oxide (NO) synthase (eNOS), leading to the production of NO, a key modulator of vascular tone (5, 8, 27). Significantly, AMPK preserves EC function during periods of metabolic and inflammatory stress. AMPK suppresses reactive oxygen production and apoptosis in ECs exposed to high concentrations of glucose or free fatty acids and protects against oxidative EC injury (6, 20, 23, 24, 35). Furthermore, AMPK preserves EC viability during anoxia and is essential for angiogenesis in response to hypoxia (6, 29, 30). AMPK also exerts potent antiinflammatory effects by inhibiting TNF-α-mediated activation of NF-κB, the expression of adhesion receptors and chemokines, and leukocyte adhesion to ECs (12, 18). Moreover, we (14, 15) recently reported that AMPK activation limits postischemic leukocyte rolling and adhesion in the venular endothelium of mice. Although AMPK plays a critical role in promoting EC function during metabolic and inflammatory stress, the underlying mechanism responsible for these vasoprotective actions is not fully understood.

Heme oxygenase (HO)-1 is a highly inducible enzyme that degrades heme into equimolar amounts of carbon monoxide (CO), iron, and biliverdin (10). This oxidative reaction is inhibited by various metalloporphyrins, including tin protoporphyrin-IX (SnPP). The induction of HO-1 in vascular ECs...
serves an important cytoprotective role by catabolizing prooxidant heme to the antioxidant bile pigments biliverdin and bilirubin and by upregulating the expression of ferritin, which exerts an additional antioxidant effect by chelating iron. In addition, the generation of bilirubin and CO by HO-1 exerts potent antiapoptotic, anti-inflammatory, and angiogenic effects in ECs (2, 9, 10, 23).

Based on the findings that AMPK and HO-1 have similar effects on EC biology, we tested whether AMPK activation is functionally linked to HO-1 gene expression in the vascular endothelium. In particular, we examined whether AMPK modulates HO-1 gene expression in human arterial and venous ECs and human arterial smooth muscle cells (SMCs). To verify our in vitro experiments with cultured vascular cells, we also investigated whether the in vivo activation of AMPK influences the expression of HO-1 in rat carotid arteries. In addition, we identified the signaling pathway by which AMPK regulates HO-1 expression and determined whether HO-1 mediates the antiapoptotic effect of AMPK on ECs.

MATERIALS AND METHODS

Materials. Streptomyacin, penicillin, trypsin, SDS, gelatin, heparin, and actinomycin D, cycloheximide, cartilage, propidium iodide, compound C, AICAR, methyl-L-arginine, Triton X-100, and meprisin, streptomycin, penicillin, trypsin, SDS, gelatin, heparin, and 100 U/ml penicillin and streptomycin in an atmosphere of 95% air-5% CO2 (23). HASMCs were grown in M199 containing heparin, and 100 U/ml penicillin and streptomycin in an atmosphere of 95% air-5% CO2 (23). HASMCs were grown in M199 (Dowex 50W-X8), and neutrally charged L-citrulline was eluted and for some experiments, a plasmid expressing a dominant negative Nrf2 mutant (1 μg/ml) that had its transactivation domain deleted was used. All constructs were generously provided by Dr. Jawed Alam (The Ochsner Clinic Foundation, New Orleans, LA). A plasmid encoding Renilla luciferase (hRluc/TK, 0.02 μg/ml) that had its transactivation domain deleted was used. 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1.0 ml/kg ip, Butler Schein Animal Health, Dublin, OH), the left carotid artery was isolated, and a local polymer-based delivery system was used to administer AICAR to the vessel wall, as we have previously described (33). The delivery system consisted of 200 μl of a 25% copolymer gel (PLF127, BASF, Florham Park, NJ) containing AICAR (1 mg) that was applied in a circumferential manner to the exposed adventitia of the carotid artery. A separate group of animals received an empty gel. After 24 h, animals were euthanized, and the left common carotid artery was excised, pulverized in liquid nitrogen, and processed for Western blot analysis. This investigation conformed with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996) and was approved by the Institutional Animal Care and Use Committee of the University of Missouri.

Statistics. Results are expressed as means ± SE. Statistical analyses were performed using a Student’s two-tailed t-test or with ANOVA with post hoc Bonferroni testing when multiple groups were compared. P values of <0.05 were considered statistically significant.

RESULTS

Treatment of HUVECs with AICAR (0.1–1.0 mM) stimulated a concentration- and time-dependent increase in HO-1 protein. An increase in HO-1 protein was detected after 8 h with 0.1 mM AICAR, and higher concentrations of AICAR showed a further increase in HO-1 protein (Fig. 1A). The induction of HO-1 protein was delayed, with a significant increase in HO-1 protein first detected at 4 h, peaked at 8 h, and returned toward baseline after 24 h of treatment (Fig. 1B). The induction of HO-1 protein by AICAR was associated with a concentration- and time-dependent rise in HO-1 mRNA that preceded the increase in HO-1 protein (Fig. 1, C and D). An increase in HO-1 mRNA was detected 1 h after AICAR exposure; transcript levels peaked between 4 and 8 h and remained elevated after 24 h of exposure. AICAR also stimulated a rise in HO-1 expression in HAECs (Fig. 2) and HASMCs (data not shown). Moreover, the perivascular administration of AICAR resulted in a significant increase in HO-1 protein expression in rat carotid arteries (Fig. 3).

Treatment of HUVECs with AICAR (0.5 mM) resulted in the activation of AMPK, as reflected by the phosphorylation of AMPK and the AMPK substrate ACC (Fig. 4A). However, the AMPK inhibitor compound C (41) blocked the AICAR-induced phosphorylation of AMPK and ACC (Fig. 4A). Similarly, the adenosine kinase inhibitor 5′-iodotubercin (19), which blocks the intracellular conversion of AICAR to 5- aminoimidazole-4-carboxamide (required for AMPK activation), abrogated the activation of AMPK by AICAR (Fig. 4A). Incubation of cells with either compound C or 5′-iodotubercin prevented the AICAR-mediated induction of HO-1 mRNA and protein (Fig. 4, B–E). Interestingly, compound C stimulated a
A significant increase in HO-1 expression in the absence of AICAR, suggesting possible nonspecific actions by this inhibitor (Fig. 4, B and C). To further confirm the involvement of AMPK in the induction of HO-1 by AICAR, we knocked down the expression of both catalytic \( \alpha \)-subunits of AMPK. Treatment of HUVECs with AMPK-\( \alpha \)-siRNA resulted in a 75% decline in AMPK-\( \alpha \)-protein (Fig. 4F). Moreover, the knockdown of AMPK-\( \alpha \) suppressed basal HO-1 protein expression and abolished the induction of HO-1 protein by AICAR. In contrast, the nontargeting siRNA failed to block AMPK-\( \alpha \) or HO-1 protein expression.

The ability of AMPK to induce HO-1 was also corroborated using the highly selective AMPK activator A-769662. Treatment of HUVECs with the AMPK activator A-769662 resulted in a pronounced increase in the expression of HO-1 mRNA (Fig. 5A). In addition, infection of HUVEC with AdAMPK-CA but not AdGFP stimulated AMPK activity and HO-1 mRNA expression (Fig. 5, B and C). Since AMPK results in the activation of eNOS and liberation of NO (5, 8, 27), which is a well-established inducer of HO-1 (26), the involvement of eNOS in the induction of HO-1 was also investigated. Consistent with previous reports (8, 27), we found that AICAR stimulated eNOS activity, and this could be prevented by the eNOS inhibitor methyl-L-arginine (Fig. 5D). However, treatment of HUVECs with methyl-L-arginine failed to inhibit the induction of HO-1 by AICAR (Fig. 5E).

In subsequent experiments, we further explored the mechanism by which AMPK induces HO-1 gene expression. Incubation of HUVECs with the transcriptional inhibitor actinomycin D blocked the induction of HO-1 mRNA and protein by AICAR (Fig. 6, A and B). In contrast, the protein synthesis inhibitor cycloheximide had no effect on the increase in HO-1 mRNA, whereas it completely suppressing the elevation in HO-1 protein induced by AICAR. Actinomycin D also attenuated the basal expression of HO-1 mRNA, whereas cycloheximide significantly increased the basal HO-1 message. Both actinomycin D and cycloheximide reduced HO-1 protein levels in untreated cells.

To determine whether the increased expression of HO-1 in response to AICAR involves the transcriptional activation of the gene encoding this protein, HUVECs were transiently transfected with a HO-1 reporter construct and promoter activity was monitored. Treatment of HUVECs with AICAR stimulated a threefold increase in HO-1 promoter activity that was abolished by mutating the AREs, suggesting that AICAR activates HO-1 gene transcription via AREs (Fig. 7A). Since the transcription factor Nrf2 plays a predominant role in ARE-mediated gene transcription, we determined whether Nrf2 mediates the activation of HO-1 by AICAR. Transfection of HUVECs with a dominant negative mutant of Nrf2 that had its activation domain deleted inhibited the AICAR-mediated increase in HO-1 promoter activity. In addition, AICAR resulted in a rapid concentration-dependent increase in Nrf2 protein beginning 1 h after drug treatment (Fig. 7, B and C). Significantly, the AICAR-mediated increase in Nrf2 protein expression was blocked by 5'-iodotubercidin, indi-

**Fig. 2.** AICAR stimulates HO-1 protein and mRNA expression in human aortic ECs. A: time course of HO-1 protein expression after the administration of AICAR (0.5 mM). B: time course of HO-1 mRNA expression after the administration of AICAR (0.5 mM). HO-1 protein and mRNA expression were quantified by scanning densitometry, normalized with respect to \( \beta \)-actin or 18S rRNA, respectively, and expressed relative to that of control, untreated cells. Results are means ± SE of 3 experiments. *Statistically significant effect of AICAR.

**Fig. 3.** AICAR stimulates HO-1 protein expression in blood vessels. HO-1 protein expression in rat carotid arteries treated with an empty gel or AICAR (1 mg)-containing gel for 24 h is shown. HO-1 protein expression was quantified by scanning densitometry, normalized with respect to \( \beta \)-actin, and expressed relative to that of control, untreated cells. Results are means ± SE of 4 measurements. *Statistically significant effect of AICAR.

**Fig. 4.** A: time course of HO-1 protein expression after the administration of AICAR (0.5 mM). B: time course of HO-1 protein expression after the administration of AICAR (0.5 mM). HO-1 protein expression was quantified by scanning densitometry, normalized with respect to \( \beta \)-actin, and expressed relative to that of control, untreated cells.
cating that AMPK activity was necessary for the activation of Nrf2 (Fig. 7D).

In a final series of experiments, the functional significance of the induction of HO-1 by AMPK was investigated. Treatment of HUVECs with TNF-α (100 ng/ml) in the presence of ceramide (25 μM), which sensitizes human ECs to TNF-α-mediated apoptosis (40), resulted in a significant decline in the number of viable cells and stimulated a marked increase in the activity of the proapoptotic effector caspase-3 (Fig. 8, A and B). Interestingly, pretreatment with AICAR (0.5 mM) for 8 h inhibited the cytokine-mediated loss of cell viability. However, the HO inhibitor SnPP reversed the cytoprotective property of AICAR, whereas the eNOS inhibitor methyl-L-arginine had no effect (Fig. 8A). In the absence of cytokine, AICAR, SnPP, or methyl-L-arginine had no effect on cell viability (Fig. 8A). AICAR also blocked cytokine-induced caspase-3 activation, and this was prevented by transfection of HUVECs with HO-1 siRNA, whereas the nontargeting siRNA had no effect (Fig. 8B). In the absence of cytokine, AICAR (Fig. 8B), HO-1 siRNA, or nontargeting siRNA (data not shown) had no effect on caspase-3 activity. Treatment of HUVECs with HO-1 siRNA abolished the induction of HO-1 by AICAR (Fig. 8C). In contrast, the nontargeting siRNA had no effect on AICAR-mediated increases in HO-1 protein expression, confirming the efficacy and selectivity of the HO-1 knockdown approach.

DISCUSSION

In the present study, we identified AMPK as a novel inducer of HO-1 gene expression in the human vascular endothelium. Induction of HO-1 was observed after pharmacological or molecular activation of AMPK and was detected in both arterial and venous ECs. The AMPK-mediated induction of HO-1 occurred in an eNOS-independent fashion and was mediated by the Nrf2/ARE signaling pathway. Significantly, we also found that activation of AMPK inhibits cytokine-mediated EC death and caspase-3 activation and that HO-1 mediates this cytoprotective effect. Thus, the ability of AMPK to induce HO-1 gene expression may provide an important mechanism by which this kinase represses EC death during periods of metabolic and inflammatory stress.

Treatment of ECs with AICAR stimulates a concentration- and time-dependent increase in HO-1 protein and mRNA expression. The induction of HO-1 by AICAR is dependent on AMPK activity since two distinct pharmacological inhibitors of AMPK activation prevented the increase in HO-1 gene expression. Furthermore, knockdown of AMPK-α1/2 blocks the induction of HO-1 by AICAR. The ability of AMPK to stimulate HO-1 expression was also confirmed using the more selective AMPK activator A-769662 and by directly infecting ECs with an adenovirus expressing constitutively active AMPK-α1, the predominant isoform of AMPK present in human ECs (13, 43). However, the induction of HO-1 after...
AMPK activation is not restricted to the vascular endothelium. Aside from stimulating HO-1 expression in HUVECs and HAECs, activation of AMPK increases HO-1 expression in HASMCs. Significantly, the induction of HO-1 by AMPK was also observed in carotid arteries after the in vivo administration of AICAR, verifying our in vitro experiments with cultured vascular cells. Our finding that AMPK induces HO-1 expression in human vascular cells and rat arteries is consistent with a previous study (4) showing that AICAR stimulates HO-1 protein expression in rat pancreatic β-cells but contrasts with another study (35) where AICAR failed to induce HO-1 in porcine aortic ECs. Disparate results between these studies may reflect differences between animal species, culture conditions, and/or AICAR exposure times. Interestingly, several therapeutic agents that activate AMPK, including peroxisome proliferator-activated receptor agonists, statins, cilostazol, resveratrol, and curcumin, are known inducers of HO-1, raising the possibility that AMPK functions as a central signaling kinase to trigger HO-1 gene expression (42). Finally, the discovery that AMPK stimulates HO-1 expression adds to a growing list of antioxidant enzymes, including manganese SOD, catalase, γ-glutamylcysteine synthase thioredoxin, and mitochondrial uncoupling protein-2, that are induced by this kinase and highlights a critical role for AMPK in maintaining endothelial redox balance (6, 23, 24, 39).

The induction of HO-1 by AMPK is dependent on de novo RNA synthesis, as the transcriptional inhibitor actinomycin D blocked the induction of HO-1 mRNA. In contrast, stimulation of HO-1 mRNA was maintained in the presence of cycloheximide, indicating that AMPK-mediated HO-1 gene expression is independent of protein synthesis. Since AMPK activates eNOS and releases NO (5, 8, 27), which is a known inducer of HO-1 (26), we initially determined the involvement of this enzyme in mediating the induction of HO-1. Although the eNOS inhibitor methyl-L-arginine blocked the activation of eNOS by AICAR, methyl-L-arginine failed to prevent the induction of HO-1, indicating that AICAR-mediated increases in HO-1 expression are independent of eNOS activation. Transient luciferase reporter assays demonstrated that AMPK directly stimulates HO-1 promoter activity. However, the induction of HO-1 gene transcription requires the presence of AREs, since mutation of this responsive element abolished the stimulation of promoter activity by AMPK. While several transcription factors can bind to AREs, recent work has indicated that Nrf2 plays a predominant role in ARE-dependent HO-1 gene expression (1). In support of this, we observed that
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AMPK stimulates a rapid rise in Nrf2 protein. The induction of Nrf2 expression by AICAR requires AMPK activity since it was prevented by 5′-iodotubercidin. Furthermore, transfection of ECs with a dominant negative Nrf2 construct abolished the activation of HO-1 promoter activity in response to AICAR. Thus, the mobilization of Nrf2 plays an integral role in mediating HO-1 gene transcription by AMPK. The ability of AMPK to activate Nrf2 provides a novel pathway by which AMPK can regulate mammalian gene transcription. However, the mechanism by which AMPK activates Nrf2 is not known. Activation of Nrf2 is regulated by the cytosolic protein Keap1, which facilitates the degradation of Nrf2 via the proteasome. While AMPK may directly phosphorylate Nrf2, phosphorylation of Nrf2 plays a limited role in modulating Nrf2-dependent gene expression (37). Alternatively, AMPK has recently been shown to inhibit proteasomal activity, and this can result in the activation of Nrf2 (38). Consistent with this notion, proteasome inhibitors have been demonstrated to stimulate HO-1 gene expression by activating Nrf2 (36).

Recent studies have indicated that AMPK serves an important survival function in ECs. Activation of AMPK inhibits EC apoptosis in response to oxidative and metabolic stress and anoxia (6, 20, 23, 24, 30, 35). In the present study, we extend this concept to show that AMPK also protects ECs from inflammatory stress. In particular, activation of AMPK by AICAR attenuated EC death and caspase-3 activation in response to TNF-α and ceramide. Interestingly, the cytoprotection afforded by AICAR is mediated by HO-1, since inhibition of HO activity or deletion of HO-1 abolished the cytoprotective effect of AMPK. The cytoprotective action of HO-1 in ECs is likely mediated by CO since scavenging of CO with hemoglobin reversed the antiapoptotic effect of HO-1, whereas the exogenous administration of this gas mimicked the protection induced by HO-1 in response to a wide variety of proapoptotic stimuli, including TNF-α (2, 28). In contrast, inhibition of eNOS activity failed to reverse the protection afforded by AICAR. This latter finding is in agreement with a recent report (29) showing that AMPK protects anoxic ECs from apoptosis in an eNOS-independent fashion. Interestingly, while we show that AMPK stimulates the expression of HO-1, a recent report (32) found that the HO-1 reaction product CO activates AMPK in ECs, raising the possibility that a positive feedback loop exists between HO-1 and AMPK that may serve to amplify the cytoprotective effect of AMPK.

The ability of AMPK to stimulate HO-1 gene expression may be of pharmacological and therapeutic significance. Given our current finding that HO-1 contributes to the antiapoptotic action of AICAR, the induction of HO-1 by AICAR may underlie its ability to inhibit ischemia-reperfusion injury (22). Furthermore, we (14) recently reported that AICAR preconditioning prevents posts ischemic leukocyte rolling along and adhesion to the murine postcapillary venular endothelium in a manner that is dependent on HO activity, suggesting that HO-1 may also contribute to the antiadhesive actions of AICAR. Moreover, since HO-1 is able to normalize EC function and blood pressure under various pathological settings (10), the induction of HO-1 may also contribute to the restoration of endothelial function and blood pressure that is observed after chronic AMPK activation by AICAR in animal models of insulin resistance and hypertension (3, 35). Finally, our finding that AMPK upregulates HO-1 expression in vascular SMCs may also be of pharmacological relevance. In particular, the induction of HO-1 may participate in the AICAR-mediated inhibition of neointima formation after arterial injury given that HO-1 is a potent inhibitor of vascular SMC growth (3, 10, 34). Thus, the induction of HO-1 may contribute to the pleiotropic beneficial effects of AMPK activators in the circulation.

The present in vitro study provides strong evidence that AMPK promotes EC viability through the induction of HO-1. However, caution is required when extrapolating findings with cultured vascular cells to the intact animal. It will be important to confirm our findings using animal models of metabolic and/or inflammatory stress. In this regard, future experiments using HO-1-deficient mice will prove indispensable in establishing a role for HO-1 in mediating the biological actions of AMPK. Moreover, these animals may provide further insights into the degree and physiological significance of possible reciprocal cross-talk between the AMPK and HO-1 systems.

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Fig. 6. AICAR-mediated HO-1 gene expression requires de novo RNA synthesis. A: effect of actinomycin D (Act D; 2 μg/ml) or cycloheximide (CX; 5 μg/ml) on the AICAR (0.5 mM for 4 h)-mediated increase in HO-1 mRNA. B: effect of Act D (2 μg/ml) or CX (5 μg/ml) on HOCl (0.5 mM for 8 h)-mediated increases in HO-1 protein. HO-1 protein or mRNA was quantified by scanning densitometry, normalized with respect to β-actin or 18S rRNA, respectively, and expressed relative to that of control, untreated cells. Results are means ± SE; n = 4. *Statistically significant change in HO-1 expression.
However, the use of highly integrated pathophysiological animal models introduces further complexity. Aside from inducing AMPK activity, stimuli arising from metabolic stress may also directly induce HO-1 expression independent of AMPK. Here again, the use of animal models that lack the requisite proteins will allow one to discern the biological effects of AMPK and HO-1 as well as their regulatory interactions.

In conclusion, the present study demonstrates that AMPK induces HO-1 gene expression in human vascular cells and rat arteries via the Nrf2/ARE pathway. In addition, we found that proteins will allow one to discern the biological effects of AMPK and HO-1 as well as their regulatory interactions.

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AMPK inhibits cytokine-mediated EC apoptosis in an HO-1-dependent fashion. The ability of AMPK to stimulate HO-1 gene expression may provide a novel mechanism by which this kinase preserves EC viability and function during periods of metabolic and inflammatory stress.

REFERENCES


