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Bradykinin stimulates endothelial cell fatty acid oxidation by CaMKK-dependent activation of AMPK

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Abstract

Endothelial cell lipotoxicity mediated by accumulation of free fatty acids is an early event in the pathogenesis of atherosclerosis. The energy-sensor AMP-activated protein kinase (AMPK) is a key regulator of endothelial cell lipid metabolism. To test the hypothesis that bradykinin (BK) regulates AMPK and fatty acid oxidation in endothelium, stimulations of bovine aortic endothelial cells (BAECs) with bradykinin were performed. BK stimulation caused a 2.3-fold increase in AMPK activity ($p < 0.05$). Activation of AMPK by BK in BAECs was inhibited by STO-609, an inhibitor of calmodulin-dependent kinase kinase (CaMKK), which is a known kinase upstream of AMPK. BK stimulation of BAECs also increased phosphorylation of acetyl-CoA carboxylase and this was inhibited by both STO-609 and over expression of an adenovirus encoded AMPK dominant negative (Ad-AMPK-DN). Furthermore, BK caused a 1.7-fold increase in palmitate oxidation in BAECs ($p < 0.05$) and this increase was completely inhibited by the Ad-AMPK-DN ($p < 0.005$). Inhibition of AMPK activation in response to BK by STO-609 had no effect on activating phosphorylation of endothelial nitric oxide synthase (eNOS) at Ser^{1177}, consistent with CaMKK and AMPK not being required for phosphorylation of eNOS in response to BK. In conclusion, BK stimulates endothelial cell fatty acid oxidation by CaMKK-dependent activation of AMPK. The effect of BK on endothelial lipid metabolism represents a novel pathway for targeting fatty acid mediated endothelial cell dysfunction.

Keywords: Bradykinin; AMPK; CaMKK; Fatty acid oxidation; eNOS

1. Introduction

Accumulation of lipids and fatty acids within endothelium is an important event in the pathogenesis of endothelial dysfunction and atherogenesis [1,2]. Intracellular accumulation of fatty acids leads to synthesis of diacylglycerol, thereby causing activation of protein kinase C, which results in impaired endothelial function by several mechanisms, including excess production of superoxide anions [2,3]. AMP-activated protein kinase (AMPK) is a heterotrimeric ($\alpha\beta\gamma$) energy-sensor that has a critical role in the regulation of fatty acid metabolism in endothelial cells [4]. Activation of AMPK stimulates fatty acid oxidation by phosphorylation of acetyl-CoA carboxylase (ACC), which reduces cellular levels of malonyl-CoA, thereby removing tonic inhibition of mitochondrial fatty acid uptake by carnitine palmitoyl transferase 1 (CPT1) [4]. Phosphorylation of ACC by AMPK also inhibits fatty acid synthesis, thereby reducing cellular accumulation of diacylglycerol [5] and triglyceride [1].

In obese rats, AMPK activity is reduced in aortic endothelium and activation of AMPK reduces triglyceride

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accumulation in endothelial cells and restores endothelial cell function [1]. Furthermore, AMPK has been implicated in various important aspects of endothelial cell biology; including stimulation of angiogenesis [6], suppression of apoptosis [7] and regulation of nitric oxide synthesis [8,9]. AMPK is reported to be regulated in endothelial cells by various stimuli including adiponectin [10], α-lipoic acid [1], the anti-diabetic drug metformin [11], extracellular nucleotides [12] and thrombin [13]. In obese patients with type 2 diabetes metformin reduces the incidence of death and myocardial infarction by a mechanism independent of glucose lowering [14], and it has been hypothesized that this might be explained by activation of endothelial cell AMPK [3]. Furthermore, endothelial cell activation of AMPK has been proposed to contribute to the beneficial vascular effects of the cholesterol lowering statin drugs [15]. Overall, however, the regulation and functions of AMPK in endothelium are incompletely understood. Of interest, the relationship between endothelial cell AMPK and the kallikrein–kinin system, which plays a critical role in the regulation of endothelial cell function, has not been adequately studied.

Bradykinin (BK) is the effector molecule of the kallikrein–kinin system and has numerous important functions in the regulation of endothelial function, including the regulation of vasodilatation, fibrinolysis and vascular permeability [16]. In a previous study, we reported that BK stimulated the phosphorylation of AMPK (αThr^{172}) in endothelial cells and also increased phosphorylation of key substrates ACC at Ser^{79} and endothelial nitric oxide synthase (eNOS) at Ser^{1177} [17]. Significantly, however, the details of the pathway by which BK activates AMPK have not been described. It is also not known whether activation of AMPK by BK regulates endothelial cell lipid metabolism. The present study tests the hypothesis that BK stimulates endothelial cell fatty acid oxidation via an AMPK mediated pathway, by studying the effects of BK on bovine aortic endothelial cells (BAECs) infected with adenovirus encoding an AMPK dominant negative (Ad-AMPK-DN). Furthermore, the mechanisms by which BK activates endothelial cell AMPK are characterized.

2. Methods

2.1. Materials and reagents

Bradykinin, HOE-140, STO-609 and L-NAME were purchased from Sigma–Aldrich (St. Louis, MO, USA). Rabbit polyclonal antibodies against α1-AMPK, $pThr^{172}$α-AMPK, p-ACC-Ser^{79} and p-eNOS-Ser^{1177} were produced as previously described [17,18]. Rabbit polyclonal antibodies against eEF2 (total) and p-eEF2-Ser^{56} were purchased from Cell Signaling Technology (Beverley, MA, USA). A mouse monoclonal antibody against eNOS was purchased from BD Transduction Laboratories (Lexington, KY, USA). Secondary antibodies (swine-anti-rabbit-HRP, rabbit-anti-mouse-HRP) were purchased from Dako (Carpinteria CA, USA). Protein A–HRP was purchased from Amersham Pharmacia (Uppsala, Sweden).

2.2. Cell culture and adenovirus

BAECs were isolated from a segment of bovine aorta (obtained from a local abattoir) and passaged from primary cultures as previously described [17]. Experiments were performed on cells grown to confluence in 9 cm diameter plastic dishes. Prior to stimulations BAECs were incubated in serum-free DMEM for 2 h, which we have previously demonstrated does not alter basal phosphorylation of AMPK or eNOS[17]. Cell lysis was performed with 0.5 ml of ice-cold cell lysis buffer (50 mM Hepes, pH 7.5, 2 mM EDTA, 50 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM dithiothreitol, 1% nonidet P-40, 10 μg/ml trypsin inhibitor, 10 μg/ml aprotinin and 1 mM PMSF) for 30 min and the lysate was clarified by centrifugation (16,000 × *g*, 5 min, 4 °C) and then stored at −80° C. The protein concentration of lysates was measured using the Bio-Rad reagent system (Bio-Rad, Hercules, CA).

For experiments involving adenovirus, BAECs were grown to ∼50% confluence and infected with adenovirus containing a control vector (Ad-null) or a dominant-negative mutant of the α1 subunit of AMPK (Ad-AMPK-DN) as previously described [19]. Adenoviral expression level was determined by visual examination of green fluorescent protein under ultraviolet light. Forty-eight hours post-infection of ∼85% of all BAECS were infected.

2.3. Immunoprecipitation of AMPK and partial purification of eNOS by adenosine diphosphate–sepharose

AMPK was immunoprecipitated by mixing 3 μl α1-AMPK antibody (1 mg/ml) with BAEC lysate (0.5–1 mg) at 4 °C for 1 h. Immunocomplexes were precipitated by mixing the sample with Protein A beads (20 μl) (Amersham Pharmacia, Uppsala, Sweden) at 4 °C for 30 min. ADP–sepharose extraction to partially purify eNOS from BAEC lysate (0.5–1 mg) were performed by mixing 20 μl of 2′5′ADP–sepharose beads (Amersham Pharmacia) with the lysate sample at 4 °C for 90 min as previously described [20]. After immunoprecipitation or ADP–sepharose extraction the beads were collected by centrifugation and washed three times in ice-cold wash buffer (1% NP40 in PBS) and once in cold PBS. Reducing Laemmli sample buffer (20 μl) was added to the beads, which were then heated to 95 °C for 4 min prior to analysis by SDS-PAGE and Western blot.

2.4. Western blotting

Samples were separated by SDS-PAGE and electrically transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA) at 30 V overnight. The membrane was blocked in 5% casein in Tris-buffered saline (TBS) for 1 h and