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Metformin represses glucose starvation induced autophagic response in microvascular endothelial cells and promotes cell death

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ABSTRACT

Metformin, the most frequently administered drug for the treatment of type 2 diabetes, is being investigated for its potential in the treatment of various types of cancer; however, the cellular basis for this putative anti-cancer action remains controversial. In the current study we examined the effect of metformin on endoplasmic reticulum (ER) stress and autophagy in glucose-starved micro-vascular endothelial cells (MECs). The rationale for our experimental protocol is that in a growing tumor MECs are subjected to hypoxia and nutrient/glucose starvation that results from the reduced supply and relatively high consumption of glucose. Mouse MECs (MMECs) were glucose-starved for up to 48 h in the absence or presence of metformin (50 μM and 2 mM) and the status of ER stress, autophagic, cell survival and apoptotic markers were assessed. Activation of ER stress and autophagy was observed in glucose starved MECs as evidenced by the significant increase in the levels of ER stress and autophagic markers while accumulation of LC3B stained punctae in the MECs confirmed autophagic activation. Treatment with 2 mM metformin, independent of AMPK, significantly reversed glucose starvation induced ER stress and autophagy in MECs, but, at 24 h, did not decrease cell viability; however, at 48 h, persistent ER stress and metformin associated inhibition of autophagy decreased cell viability, caused cell cycle arrest in G2/M and increased the number of cells in the sub-G0/G1 phase of cell cycle. Treatment with metformin reduced phosphorylation of Akt and mTOR and inhibited downstream targets of mTOR. Our findings support the argument that treatment with metformin when used in combination with glycolytic inhibitors will inhibit pro-survival autophagy and promote cell death and potentially prove to be the basis for an effective anti-cancer strategy.

1. Introduction

Epidemiologic evidence suggests that treatment with metformin (1,1-dimethylbiguanide hydrochloride), the most widely prescribed oral anti-hyperglycemic/anti-diabetic drug, reduces the incidence of cancer and also improves the prognosis among subjects affected by various cancers; however, the cellular mechanism(s) whereby metformin mediates its anti-cancer effect remains controversial [1,2]. Data from *in vitro* studies show that high (millimolar) concentrations of metformin significantly inhibit cell growth in cultured cancer cells [3] and metformin is lethal when combined with glucose withdrawal/starvation or in combination with glycolytic inhibitors such as 2-deoxyglucose (2DG) [4]. High micromolar concentrations of metformin are also known to inhibit mitochondrial complex I in human endothelial cells and

Abbreviations: 2DG, 2-deoxyglucose; 3MA, 3-methyl adenine; 4E-BP1, Eukaryotic translation initiation factor 4E-binding protein 1; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AMPK, AMP activated protein kinase; ATF4, Activating transcription factor 4; Baf B1, Bafilomycin B1; CHOP, CCAAT/enhancer binding protein-homologous protein; DCA, Dichloroacetate; DDIT4/REDD1, DNA-damage-inducible transcript 4; ECs, Endothelial cells; ER, Endoplasmic reticulum; GRP78, Glucose regulated protein 78; HUVECs, Human umbilical vein endothelial cells; LC3A, Microtubule-associated protein 1 light chain 3 alpha; LC3B, Microtubule-associated protein 1 light chain 3 beta; MECs, Microvascular endothelial cells; MMECs, Mouse microvascular endothelial cells; mTOR, Mammalian target of rapamycin; Rap, Raptor; UPR, Unfolded protein response; VEGF, Vascular endothelial growth factor.

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cancer cells and hence reduce tumorigenesis [5,6]. In mitochondria, metformin inhibits the transfer of electrons from NADH to the electron transport chain thus increasing the reliance on lactate production as a means to cycle NADH back to NAD^+ and impairing mitochondrial production of ATP causing cellular energy stress [7]. Therefore, using metformin with other therapies that induce energy stress should have a synergistic effect [7]. It is argued that metformin directly or indirectly, via an increase of the AMP/ATP ratio, activates AMP-activated protein kinase (AMPK); however, the question as to whether AMPK activation is essential for all of the known therapeutic actions of metformin remains controversial [8,9]. Metformin has also been shown to induce apoptosis through AMPK-dependent inhibition of endoplasmic reticulum (ER) stress response/unfolded protein response (UPR) signaling in acute lymphoblastic leukemia [10]. Additionally, it has been reported that metformin inhibits pro-survival autophagy in prostate cancer cells [11,12]; however, metformin has also been reported to activate autophagy in cancer cells [13] and in cardiac tissue [14].

Both ER stress and autophagy are argued to be pro-survival mechanisms in cancer and are initiated in response to metabolic stressors such as glucose starvation and play important roles in supporting pathological angiogenesis and thus promote metastasis [15–19]. In cancer cells exposed to stress the autophagic response channels essential nutrients from less important cellular processes to those required for survival. Glucose starvation/2DG induced ER stress response/UPR and subsequent autophagy in cancer [20] are known to be adaptive pro-survival mechanisms [21,22] and render cancer cells resistant to chemo- and radiation therapy [23]. Therefore, targeting and inhibiting such pro-survival responses should prove detrimental to cancer cells and sensitize them to chemotherapeutic agents and radiation therapy [24,25].

Endothelial cells (ECs) play a major role in facilitating an adequate supply of oxygen and nutrients for a growing tumor [26]. Tumor ECs differ from normal ECs and are characterized by altered metabolism, a defective endothelial monolayer with large intercellular pores/channels and abnormal sprouting resulting in leaky blood vessels that promote tumor progression and metastasis [26–28]. In a growing tumor, ECs are exposed to high levels of Vascular Endothelial Growth Factor (VEGF), a potent vasodilator and endothelial growth factor, and double their glycolytic flux with a decrease in oxidative phosphorylation and exhibit characteristics of the Warburg effect, which is commonly observed in cancer cells [29]. However, although clinically approved, the pharmacological inhibition of VEGF has limited benefits in the therapeutic management of cancer as the tumors acquire resistance to a drug regimen that solely targets the VEGF pathway [27]. In such a scenario, a therapeutic strategy, which is both anti-endothelial and anti-angiogenic is predicted to be more effective [30].

Targeting the metabolism of tumor ECs with drugs, which in combination with other therapeutic strategies that cause energy stress, should have a synergistic effect. Schoors et al. reported that blocking phosphofructokinase-2/fructose-2,6-bisphosphatase 3 (PFKFB3) with 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) results in a partial and transient inhibition of glycolysis and thereby reduces pathological angiogenesis [31]. *In vitro* and *in vivo* studies have shown that a small molecule inhibitor of mono-carboxylate transporters, alpha-cyano-4 hydroxycinnamic acid sensitizes brain tumor cells to radiation therapy and decreases the invasiveness of the tumor [32,33]. Agents that promote respiration and activity of the mitochondria such as dichloroacetate (DCA) have also been reported to induce death in cancer cells both *in vitro* and *in* vivo [34]. In addition, an inhibitor of glycolysis, 2DG, has been shown to enhance the response of human neck and head cancer xenografts to cisplatin treatment [35].

The effects of metformin on glucose starvation associated ER stress and autophagy and cell fate have not been investigated in ECs. In the current study, we have examined the effect of metformin on glucose starvation induced ER stress and autophagy and subsequent cell fate in VEGF overexpressing mouse microvascular ECs (MMECs) that are capable of forming well differentiated angiosarcomas in mice [36–38].

2. Materials and methods

2.1. Chemicals, biochemicals, reagents and antibodies

All chemicals, biochemicals and reagents used, including metformin (Cat # D150959), 2-deoxyglucose (Cat # D6134) and bafilomycin B1 (Cat # 11707) were of analytical grade and purchased from Sigma-Aldrich, Inc. (MO, USA), unless otherwise stated. Primary antibodies, against GRP78 (Cat # 3177), ATF4 (Cat # 11815), LC3A (Cat # 4599), LC3B (Cat # 3868), pAMPK (T172; Cat # 2531), AMPK (Cat # 2532), pAkt (S473; Cat # 4060), Akt (Cat # 4691), pmTOR (S2448; Cat # 5536), mTOR (Cat # 2983), p4E-BP1 (T37/46; Cat # 2855), 4E-BP1 (Cat # 9644), pS6 (S235/236; Cat # 4858), pS6 (S240/244; Cat # 5364), S6 ribosomal protein (Cat # 2317), cleaved caspase-3 (Cat # 9664), caspase-3 (Cat # 9665) and β-actin (Cat # 3700) were purchased from Cell Signaling Technology, Inc. (MA, USA), and against CHOP (Cat # sc7351) was purchased from Santa Cruz Biotechnology, Inc. (TX, USA). HRP-linked secondary antibodies (anti-rabbit IgG, Cat # 7074 and anti-mouse IgG, Cat # 7076) were purchased from Cell Signaling Technology, Inc. (MA, USA). Alexa-fluor 555 conjugated secondary antibody (Cat # A-21430) for immunofluorescence studies was purchased from Invitrogen (NY, USA).

2.2. Endothelial cell culture

Mouse microvascular endothelial cells (MMECs, Cat # CRL-2460, MS1-VEGF) were purchased from American Type Culture Collection (ATCC, VA, USA) and serially passaged, for the study. MMECs (also called MS1-VEGF) were produced by overexpressing the primate VEGF-121 in the MS1 endothelial cell line (Cat # CRL-2279; ATCC, VA, USA) derived from mice pancreatic microvasculature and immortalized with temperature sensitive SV40 large T antigen [36]. MMECs have been shown to generate well-differentiated angiosarcomas in nude mice [36]. In the current study, MMECs were grown in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, NY, USA), at 11 mM glucose concentration, supplemented with 5% FBS (Sigma-Aldrich, MO, USA), in a humidified atmosphere with 5% CO_2 at 37 °C [39]. Primarily MMECs were used in the current study. MDA-MB 231 (human breast cancer cells; Cat # HTB-26) and human umbilical vein endothelial cells (HUVECs; Cat # PCS-100-010) were also purchased from ATCC (VA, USA) for few experiments related to autophagy.

2.3. Cell treatments

MMECs were subjected to glucose starvation/withdrawal for 24 h and 48 h in the presence or absence of metformin (50 μM and/or 2 mM) and normal glucose (11 mM) exposed cells in the presence or absence of metformin was considered as suitable normal controls. Normal glucose levels are based on established random plasma glucose measurements from non-diabetic mice [40]. 50 μM metformin was chosen based on previous studies that demonstrated that at this concentration metformin protected MMECs against hyperglycemia-induced endothelial cell senescence and, furthermore, 50 μM is within the upper level of the plasma concentration of metformin that may be expected during its therapeutic use in type 2 diabetes [39,41,42]. Concentration-dependent studies on the effect of varying concentrations (0–