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Uncoupling of eNOS contributes to redox-sensitive leukocyte recruitment and microvascular leakage elicited by methylglyoxal

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ABSTRACT

Elevated levels of the glycolysis metabolite methylglyoxal (MG) have been implicated in impaired leukocyte–endothelial interactions and vascular complications in diabetes, putative mechanisms of which remain elusive. Uncoupling of endothelial nitric oxide synthase (eNOS) was shown to be involved in endothelial dysfunction in diabetes. Whether MG contributes to these effects has not been elucidated. By using intravital microscopy *in vivo*, we demonstrate that MG-triggered reduction in leukocyte rolling velocity and increases in rolling flux, adhesion, emigration and microvascular permeability were significantly abated by scavenging reactive oxygen species (ROS). In murine cremaster muscle, MG treatment reduced tetrahydrobiopterin (BH4)/total biopterin ratio, increased arginase expression and stimulated ROS and superoxide production. The latter was significantly blunted by ROS scavengers Tempol (300 μM) or MnTBAP (300 μM), by BH4 supplementation (100 μM) or by NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 20 μM). In these tissues and cultured murine and human primary endothelial cells, MG increased eNOS monomerization and decreased BH4/total biopterin ratio, effects that were significantly mitigated by supplementation of BH4 or its precursor sepiapterin but not by L-NAME or tetrahydroneopterin, indicative of MG-triggered eNOS uncoupling. MG treatment further decreased the expression of guanosine triphosphate cyclohydrolase I in murine primary endothelial cells. MG-induced leukocyte recruitment was significantly attenuated by supplementation of BH4 or sepiapterin or suppression of superoxide by L-NAME confirming the role of eNOS uncoupling in MG-elicited leukocyte recruitment. Together, our study uncovers eNOS uncoupling as a pivotal mechanism in MG-induced oxidative stress, microvascular hyperpermeability and leukocyte recruitment *in vivo*.

1. Introduction

Inflammatory injury afflicting endothelial cells contributes to the pathophysiology of multiple vascular diseases [1]. In diabetes, chronic hyperglycemia leads to endothelial dysfunction which fosters deranged microcirculation [2,3]. The underlying mechanisms of impaired endothelial functions and innate immunity in diabetes are, however, not completely understood. Chronic hyperglycemia results in excessive levels of the cytotoxic metabolite methylglyoxal (MG) [4,5] which serves as a precursor to form advanced glycation end-products [6]. MG has been implicated in the pathogenesis of diabetes [7], neuronal injury [8] and vascular complications [9–11]. MG-induced cytotoxicity encompasses both necrotic [12] and apoptotic cell death [10] leading to alterations of muti-organ homeostasis. Moreover, MG is a powerful modulator of mitochondrial functions, thus altering both cellular energy and redox balance [13].

Inflammatory sequelae of pathological MG concentrations comprise functional modulation of various immune cells. MG was shown to stimulate cytokine induction [14], activate macrophages [15,16], suppress T-cell-mediated immune functions [17] and modulate innate immunity by enhancing neutrophil apoptosis and Mac-1 expression [11]. Recently, MG was shown to stimulate leukocyte–endothelial interactions in an *in vivo* model of acute inflammation [18]. MG treatment up-regulated NF-κB-dependent expression of endothelial cell adhesion molecules, mitigated intravascular leukocyte rolling velocity, increased rolling flux and leukocyte adhesion and accelerated leukocyte emigration in mice [18]. In diabetic mice, deranged leukocyte–endothelial interactions were documented [19], but the contribution of the increased metabolite MG alone has not been completely characterized. Mechanisms that mediate pro-inflammatory effects of elevated MG, however, are largely unidentified.

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Nitric oxide (NO) homeostasis is an important determinant of physiological cardiovascular functions and its impairment is involved in the pathophysiology of diabetes [20]. Furthermore, NO is a powerful modulator of leukocyte–endothelial interactions [21,22]. The constitutively expressed endothelial nitric oxide synthase (eNOS) catalyses the reaction for generation of NO and L-citrulline from L-arginine [23] and requires the presence of the essential cofactors tetrahydrobiopterin (BH4) and NADPH [24]. Deficiency of either L-arginine or BH4 reduces the generation of NO by eNOS which triggers superoxide ($O_2^{\bullet-}$) production, a process termed eNOS uncoupling [25]. Ramifications of eNOS uncoupling include increased eNOS monomerization, tyrosine nitration and formation of dihydrobiopterin (BH2) [26,27]. Guanosine triphosphate cyclohydrolase I (GTPCH1) is the rate-limiting enzyme that regulates *de novo* BH4 synthesis whereas dihydrofolate reductase (DHFR) reduces BH2, thus, salvaging BH4 from BH2 [28]. eNOS uncoupling-derived $O_2^{\bullet-}$ stimulates platelet aggregation [29] and increased monocyte adhesion to endothelial cells [30], both effects favouring diabetic vasculopathy. Moreover, reactive oxygen species (ROS) are potent regulators of leukocyte–endothelial interactions [31–35]. In fact, redox imbalance is known to participate in a multitude of pathophysiological processes, the mechanisms of which may in part be secondary to eNOS uncoupling.

MG influences NO and redox balance and contributes to a myriad of hyperglycemia-induced alterations of cellular functions *in vivo* [5], mechanisms of which still remain elusive. eNOS uncoupling was previously shown to be responsible for endothelial dysfunction in diabetic mice [36] and essential in both endothelial dysfunction and peripheral neuropathy in Zucker diabetic fatty rats [37]. It was only recently shown that MG affects eNOS expression or functions. On the one hand, sensitivity of eNOS to MG was increased by the upregulation of eNOS transcription [38], while on the other hand, eNOS protein expression was decreased by MG [39,40]. Discordantly, MG was reported to modulate eNOS activity without significantly affecting eNOS protein expression but by suppressing phosphorylation of eNOS at serine-1179 [41]. In preeclamptic vasculature, increased MG concentrations were shown to affect the expression of arginase and arginase-dependent lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) [42], that are associated with enhanced $O_2^{\bullet-}$ production and uncoupling of eNOS [27,42,43]. Arginase is further inhibitable by the NOS inhibitor L-NAME [44]. Whether or not MG-sensitive eNOS regulation participates in leukocyte–endothelial interactions is not known.

We hypothesized that MG-elicited leukocyte recruitment and microvascular hyperpermeability are mediated by modulation of eNOS expression or functions, a mechanism common to other MG-induced pathologies [40,42]. To reveal the presence of eNOS uncoupling and its role in MG-triggered leukocyte recruitment and microvascular hyperpermeability, we quantified BH4 reduction, eNOS monomerization, L-NAME inhibitable $O_2^{\bullet-}$ production and used fluorescence imaging and real-time intravital microscopic analysis to study microvascular permeability changes and leukocyte–endothelial interactions induced by exogenous MG.

2. Materials and methods

2.1. Mice

Male C57BL/6 mice (Charles River, Saint-Constant, QC, Canada) between 8 and 12 wk-old and young C57BL/6 mice at 5−7 day-old were used in this study. This study was carried out with the approval of animal protocols from the University Committee on Animal Care and Supply (UCACS; protocol permit # 20070028) at the University of Saskatchewan following the standards of the Canadian Association of Animal Care. All surgery was performed under ketamine/xylazine anesthesia, and all efforts were made to minimize animal suffering.

2.2. Intravital microscopy

Mice were anesthetized using an i.p. injection of 10 mg/kg xyalzine (Bayer, Toronto, ON, Canada) and 200 mg/kg ketamine hydrochloride (Rogar, Montreal, QC, Canada). The mouse cremaster muscle preparation was used to study dynamic leukocyte–endothelial interactions in microvasculature as described previously [45,46]. The cremaster muscle was superfused with 37 °C-warmed bicarbonate-buffered saline (pH 7.4; containing in mM 133.9 NaCl, 4.7 KCl, 1.2 $MgSO_4$ and 20 $NaHCO_3$, all reagents purchased from Fisher Scientific, Toronto, ON, Canada). A BX61WI Olympus upright microscope with a LUCPLFLN 20× objective lens was connected to a 3CCD colour video camera (DXC-990, Sony) for bright-field intravital microscopy. Throughout the experiment, leukocyte behaviour and hemodynamic changes in the selected cremasteric postcapillary venule (25–40 μm diameter) were visualized, projected on a TV monitor and recorded at real time on a DVD recorder. The number of rolling, adherent, and emigrated leukocytes during offline playback analysis of the recorded video was determined in the cremasteric microvasculature as described previously [18,45,46]. MG (25 or 50 mg/kg b.w.; Sigma, Oakville, ON, Canada) dissolved in saline, was administered by an intrascrotal injection for 4 h before the cremaster muscle was exposed for intravital microscopy. Where indicated, the pharmacological agents used to modulate MG-induce leukocyte recruitment were dissolved in the bicarbonate-buffered saline and superfused for 2 h on the exposed cremaster muscle. Manganese (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP, Santa Cruz, Dallas, TX, USA) or 1-oxyl-2,2,6,6-tetramethyl-4-hydroxypiperidine (Tempol, Santa Cruz) was used to suppress ROS production. NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; Sigma) was administered to inhibit uncoupled eNOS-derived $O_2^{\bullet-}$ production [47,48]. To maintain eNOS dimerization, the NOS cofactor 5,6,7,8-tetrahydroboipterin (BH4; Sigma) or the negative control, a pteridine analogue, 5,6,7,8-tetrahydroneopterin (NH4; Schircks laboratories, Switzerland) was freshly-prepared and administered. NH4 has similar antioxidant effects as BH4 but, unlike BH4, is ineffective in restoring uncoupled eNOS [49]. BH4 availability may be compromised due to oxidative transformation to 7,8-dihydrobiopterin (BH2) [50,51]. Thus, BH4- or NH4-containing superfusion buffer was replaced every 30 min by freshly prepared BH4 to maximize BH4 and minimize BH2 availability. Sepiapterin (50 μM; Cayman, Ann Arbor, MI, USA), a substrate for BH4 synthesis via the pterin salvage pathway, was used to increase BH4 levels. Sepiapterin functions as both a precursor as well as a by-product of BH4 synthesis and is also described as a BH4 analog [52].

2.3. Measurement of microvascular permeability

MG-triggered microvascular leakage was studied in postcapillary venules of the cremaster muscle as described previously [46,53,54]. Briefly, MG (100 μM) was superfused for 1 h over the exposed cremaster muscle and where indicated, BH4 (100 μM) or the ROS scavenger Tempol (300 μM) or MnTBAP (300 μM) was superfused for 30 min prior to and 1 h during MG superfusion. FITC-labelled bovine serum albumin (BSA; Sigma) at 25 mg/kg b.w. was injected to the mice i.v. at the start of the experiment, and FITC-derived fluorescence (excitation at 495 nm, and emission at 525 nm) in the venule was detected using a monochrome deep-cooled CCD digital camera (Retiga SRV, QImaging, Surrey, BC, Canada) mounted on BX61WI Olympus upright microscope. The intensity of FITC-albumin-derived fluorescence within the lumen of the venule and in the adjacent perivascular tissue was recorded and analysed using METAMORPH software (MetaMorph®, Molec-