Free Radical Biology and Medicine 110 (2017) 390–398

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original article

Examining a role for PKG Iα oxidation in the pathogenesis of cardiovascular dysfunction during diet-induced obesity

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ARTICLE INFO

Key words:
PKG Iα
Disulfide
Oxidation
NOS uncoupling
Diet-induced obesity

ABSTRACT

Background: Protein kinase G (PKG) Iα is the end-effector kinase that mediates nitric oxide (NO)-dependent and oxidant-dependent vasorelaxation to maintain blood pressure during health. A hallmark of cardiovascular disease is attenuated NO production, which in part is caused by NO Synthase (NOS) uncoupling, which in turn increases oxidative stress because of superoxide generation. NOS uncoupling promotes PKG Iα oxidation to the interprotein disulfide state, likely mediated by superoxide-derived hydrogen peroxide, and because the NO-cyclic guanosine monophosphate (cGMP) pathway otherwise negatively regulates oxidation of the kinase to its active disulfide dimeric state. Diet-induced obesity is associated with NOS uncoupling, which may in part contribute to the associated cardiovascular dysfunction due to exacerbated PKG Iα disulfide oxidation to the disulfide state. This is a rational hypothesis because PKG Iα oxidation is known to significantly contribute to heart failure that arises from chronic myocardial oxidative stress.

Methods and results: Bovine arterial endothelial cells (BAECs) or smooth muscle cells (SMCs) were exposed to drugs that uncouple NOS. These included 1,3-bis(2-chloroethyl) − 1-nitrosourea (BCNU) which promotes its S-glutathiolation, 4-diamino-6-hydroxy-pyrimidine (DAHP) which inhibits guanosine-5′-triphosphate-cyclohydrolase 2 to prevent BH_4 synthesis or methotrexate (MTX) which inhibits the regeneration of BH_4 from BH_2 by dihydrofolate reductase. While all the drugs mentioned above induced robust PKG Iα disulfide dimerization in cells, exposure of BAECs to NOS inhibitor L-NMMA did not. Increased PKG Iα disulfide formation occurred in hearts and aortae from mice treated in vivo with DAHP (10 mM in a drinking water for 3 weeks). Redox-dead C42S PKG Iα knock-in (KI) mice developed less pronounced cardiac posterior wall hypertrophy and did not develop cardiac dysfunction, assessed by echocardiography, compared to the wild-type (WT) mice after chronic DAHP treatment. WT or KI mice were then subjected to a diet-induced obesity protocol by feeding them with a high fat Western-type diet (RM 60% AFE) for 27 weeks, which increased body mass, adiposity, plasma leptin, resistin and glucagon levels comparably in each genotype. Obesity-induced hypertension, assessed by radiotelemetry, was mild and transient in the WT, while the basally hypertensive KI mice were resistant to further increases in blood pressure following high fat feeding. Although the obesogenic diet caused mild cardiac dysfunction in the WT but not the KI mice, gross changes in myocardial structure monitored by echocardiography were not apparent in either genotype. The level of cyclic guanosine monophosphate (cGMP) was decreased in the aortae of WT and KI mice following high fat feeding. PKG Iα oxidation was not evident in the hearts of WT mice fed a high fat diet.

Conclusions: Despite robust evidence for PKG Iα oxidation during NOS uncoupling in cell models, it is unlikely that PKG Iα oxidation occurs to a significant extent in vivo during diet-induced obesity and so is unlikely to mediate the associated cardiovascular dysfunction.

1. Introduction

A global obesity pandemic, resulting at least in part from altered dietary practices and a decline in physical activity, continues to emerge. Whilst developmental programming and genetic predisposition influence the propensity for obesity [1], a diet high in calories is a major determinant. Obesity is an established risk factor for type-2 diabetes, cancer and cardiovascular diseases [2]. In terms of the cardiovascular system, obesity is associated with atherosclerosis [3], blood vessel dysregulation and hypertension [4], as well as maladaptive changes to the myocardium that alters its structure and compromises its function [5,6].

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http://dx.doi.org/10.1016/j.freeradbiomed.2017.07.007
Received 17 January 2017; Received in revised form 13 June 2017; Accepted 5 July 2017
Available online 06 July 2017

Protein kinase G (PKG) is the end-effector kinase that mediates nitric oxide (NO)-dependent vasorelaxation [7], with the Iα isoform mediating oxidant-dependent vasorelaxation to maintain normal healthy blood pressure [8,9] and regulate diastolic relaxation [10]. Whilst these finding support a role for oxidant-activated PKG Iα in the maintenance of health, oxidation of this kinase have also been observed in the pathogenesis of cardiovascular disease. PKG Iα oxidation contributed to sepsis-induced hypotension [11], as well as myocardial stress-induced apoptosis [12] and cardiac hypertrophy resulting from pressure overload induced by transverse aortic constriction [13]. Consistent with cyclic guanosine monophosphate (cGMP) negatively regulating PKG Iα [14,15], scenarios in which this second messenger is reduced, such as when NO is less bioavailable, may potentiate oxidation of the kinase to perhaps causatively mediate dysfunction [12]. Consistent with these ideas, pharmacological agents that elevate cGMP or mice expressing C42S PKG Iα that is resistant to disulfide formation [12,13] demonstrate cardioprotection.

Obesity is associated with endothelial dysfunction, leading to reduced levels of NO and consequentially cGMP, which may causatively manifests as impaired blood vessel relaxation and hypertension [16]. The lower levels of NO is thought to be significantly mediated by 'uncoupling' of NO synthase (NOS) resulting from depletion of cofactors such as tetrahydrobiopterin (BH_4) [17]. Such uncoupling is further exacerbated by the concomitant accumulation of BH_2 competing with BH_4 for its NOS binding site, resulting in superoxide production [17,18]. Dismutation of superoxide to hydrogen peroxide (H_2O_2) can oxidise and so activate PKG Iα via disulfide formation. Consistent with this, obesity results in eNOS uncoupling in mice [19], and oxidative stress in humans [20]. Oxidative stress can also promote eNOS uncoupling by an additional mechanism involving S-glutathionylation of its reductase domain [21]. Thus NOS uncoupling has a two-fold, synergistic impact on PKG Iα oxidation. This is because uncoupling not only results in an elevation in oxidants that may target the kinase, but the associated loss of NO will lower cGMP levels that otherwise negatively regulate PKG Iα disulfide formation.

Based on the considerations above, disulfide PKG Iα is likely to occur during times of NOS uncoupling such as obesity, and because of the important roles of this kinase in the cardiovascular system, its oxidation is anticipated to have functional consequences that may or may not be maladaptive. In the current study, we tested the rational hypothesis that potentiated PKG Iα oxidation occurs in vivo during scenarios in which NOS is uncoupled such as obesity, utilising a high fat feeding as a model. We also performed complementary investigations using well-established pharmacological agents to monitor oxidation of the kinase in cell, as well as in vivo, models. By comparing the response of transgenic knock-in (KI) mice expressing C42S PKG Iα, which is fully resistant to inter-chain disulfide formation at cysteine 42, to wild-type (WT) we sought to define the impact of obesity-induced PKG Iα oxidative activation on cardiovascular function.

2. Materials and methods

2.1. Cell experiments and studies with eNOS uncoupling agents in mice

Cells were grown on 12-well plates in an incubator at 37 °C with a 95% O_2:5% CO_2 environment. Bovine aortic endothelial cells (BAECs) that are constitutively deficient in PKG Iα were transfected with WT or C42S kinase DNA as described [12]. Once confluent, BAECs and primary rat aortic smooth muscle cells (SMCs) were treated with methotrexate (MTX, Santa Cruz Biotechnology, UK), 1,3-Bis(2-chloroethyl) − 1-nitrosourea (BCNU), 2,4-diamino-6-hydroxypyrimidine (DAHP), N^G-monomethyl-L-arginine acetate salt (L-NMMA) or H_2O_2 (all from Sigma, UK) for 3 or 6 h.

Immunoblotting analysis of the redox state of PKG Iα was performed as described previously [8,11,22] with maleimide (100 mM) used in preparation buffers to alkylate thiols and so limit thiol disulfide exchange. Antibodies used in these studies included cGKIα (E-17; Santa Cruz Biotechnology) or PKG antibody (ADI-KAP-PK005; Enzo Life Science). Horseradish peroxidase–linked secondary antibody (Dako) and ECL reagent (GE Healthcare) were used. Digitized immunoblots were analysed quantitatively with a Gel-Pro Analyzer 3.1.

All procedures were performed in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 in the UK. C42S PKG Iα KI mice constitutively expressing PKG Iα Cys42Ser were generated by Taconic Artemis and maintained on a C56BL/6 as described before [8,11,22]. Only male mice were used in the study. In some experiments, age and body-weight matched C56BL/6 male mice were purchased from Charles River, UK. Animals had ad libitum access to standard chow and water and were kept in a specific pathogen-free conditions under a 12-h day/night cycle at 20 °C and 60% humidity. DAHP (10 mM) was administered in the drinking water of C57BL/6 mice (n = 4/group) for 3 weeks. The water containing DAHP was refreshed every other day. At the end of the protocol, mice were euthanized and hearts and vessels were collected for measurement of the redox status of PKG Iα as described above. In a separate set of experiments C57BL/6 mice were implanted with radiotelemetry transmitters (as described below) and blood pressure was measured for three days to establish the baseline, after which DAHP was administered in the drinking water for a further 3 weeks. In an additional set of experiments, WT or KI mice were given DAHP in their drinking water for 6 weeks, monitoring cardiac function by non-invasive echocardiography, as described below, basally before the drug treatment and then at 1, 3 and 6 weeks afterwards.

2.2. High fat feeding study in mice

Age and body-weight matched WT or KI male mice were employed in this study. WT or KI male mice were fed a control diet of standard chow pellets after weaning (n = 10/group) and had ad libitum access to water throughout the study. At the age of 10 weeks mice were implanted with radiotelemetry transmitters, as described below, and then kept in individual cages enabling long-term acquisition of telemetric data, as well as accurate measurement of body weight and food intake. After the baseline cardiovascular parameters were recorded, WT or KI mice were chronically administered a control RM3 (Special Diet Services, UK) or high fat (RM 60% AFE, catalogue number 824054, LBS Serving Biotechnology Ltd, UK) diet, on a random basis (n = 5/group). This food was provided ad libitum in sturdy clear glass 60 ml receptacles with a purpose-drilled hole in the middle of the cap (Suppl. Fig. 1), designed to limit the spillage of food so that consumption could be accurately recorded. The control RM3 diet was ground, so to physically resemble the high fat diet, and was provided in an identical way as the high fat diet, with content of the containers replaced at least twice a week.

The RM 60% AFE diet consisted of 20% protein, 20% carbohydrate and 60% fat, whereas the regular chow RM3 diet comprised 20% protein, 70% carbohydrate and 10% fat. Body weight was recorded weekly for 16 weeks, after which measurements were performed on a fortnightly basis. Food intake was measured randomly at three time points at the beginning, middle or the end of the study. At the end of the feeding protocol, mice were anesthetized and subjected to the terminal non-invasive echocardiographic measurement of their cardiac function as described below. After completion of echocardiography imaging, mice were euthanized and blood was rapidly sampled from the inferior vena cava and immediately analysed for sodium (Na^+), chloride (Cl^-) and blood urea nitrogen (BUN) using an iSTAT blood biochemistry analyzer with EC8+ iSTAT cartridges (Abaxis, UK). The remaining blood was centrifuged and the plasma was stored at −80 °C until further analysis.

Hearts, aortae and the primary fat depositories were subsequently harvested. The wet mass of the peritoneal, mesenteric, perirenal and epididymal fat tissues was measured to index total body adiposity. The