Available online at www.sciencedirect.com

SCIENCE DIRECT

Free Radical Biology & Medicine 38 (2005) 755–762

www.elsevier.com/locate/freeradbiomed

Original Contribution

Involvement of mtDNA damage in free fatty acid-induced apoptosis

Valentina Grishko, Lyudmila Rachek, Sergiy Musiyenko, Susan P. LeDoux, Glenn L. Wilson*

Department of Cell Biology and Neuroscience, University of South Alabama College of Medicine, Mobile, AL 36688, USA

Received 17 August 2004; revised 12 November 2004; accepted 12 November 2004
Available online 14 December 2004

Abstract

A growing body of evidence indicates that free fatty acids (FFA) can have deleterious effects on β-cells. It has been suggested that the β-cell dysfunction and death observed in diabetes may involve exaggerated activation of the inducible form of nitric oxide synthase (iNOS) by FFA, with the resultant generation of excess nitric oxide (NO). However, the cellular targets with which NO interact have not been fully identified. We hypothesized that one of these targets might be mitochondrial DNA (mtDNA). Therefore, experiments were initiated to evaluate damage to mtDNA caused by exposure of INS-1 cells to FFA (2/1 oleate/palmetate). The results showed that FFA caused a dose-dependent increase in mtDNA damage. Additionally, using ligation-mediated PCR, we were able to show that the DNA damage pattern at the nucleotide level was identical to the one induced by pure NO and different from damage caused by peroxynitrite or superoxide. Following exposure to FFA, apoptosis was detected by DAPI staining and cytochrome *c* release. Treatment of INS-1 cells with the iNOS inhibitor aminoguanidine protected these cells from mtDNA damage and diminished the appearance of apoptosis. These studies suggest that mtDNA may be a sensitive target for NO-induced toxicity which may provoke apoptosis in β-cells following exposure to FFA.

Keywords: Free fatty acids; Mitochondrial DNA; Nitric oxide; Apoptosis; β-cell; Diabetes mellitus; Free radicals

Introduction

A common finding in many states of insulin resistance, including obesity and type II diabetes, is a frank increase in circulating and cellular free fatty acid (FFA) content [1,2]. Results reported by numerous laboratories have suggested that lipid accumulation into islet tissue is harmful for normal β-cell function, and that this elevation in FFA content ultimately leads to the depletion of β-cells by a process termed lipotoxicity. Exposure of β-cells to high concentrations of FFA has been correlated with impaired insulin secretion and changes in the expression of genes involved in the lipogenic and fat oxidation pathways [3,4]. Moreover, prolonged exposure to an excess of circulating fatty acids seems to be a key event in the initiation of apoptosis. The possibility that exposure to FFA may damage β-cells by progressively increasing total islet fat deposition has been suggested by several investigators [5–7]. It is believed that this, in turn, produces mitochondrial changes which include the uncoupling of oxidative phosphorylation and the generation of both reactive oxygen (ROS) and nitrogen (NO) species, as well as impaired endogenous antioxidant defenses, by decreasing intracellular glutathione. These effects appear to lead to diminished glucose-induced β-cell proliferation, defective insulin secretion, and increased β-cell apoptosis. Although the exact mechanisms leading to these deleterious effects have yet to be fully elucidated, it has been suggested that the β-cell dysfunction and death observed in diabetes may involve exaggerated activation of the inducible form of nitric oxide synthase (iNOS) by FFA with the resultant generation of excess NO [8]. Because intracellular NO is an important mediator of programmed cell death, it seems plausible that the loss of the β-cells observed in the course of NIDDM might be, at least

Abbreviations: FFA, free fatty acids; ROS, reactive oxygen species; iNOS, inducible nitric oxide synthase; BSA, bovine serum albumin; LM-PCR, ligation-mediated polymerase chair reaction; XO, xanthine oxidase; HRP, horseradish peroxidase; DAPI, 4,6-diamidino-2-phenylindole; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

* Corresponding author. Fax: +1 251 460 6771.
E-mail address: gwilson@usouthal.edu (G.L. Wilson).

0891-5849/$ - see front matter © 2004 Published by Elsevier Inc.
doi:10.1016/j.freeradbiomed.2004.11.023

partially, the result of NO-induced apoptosis [9]. Since NO is a highly reactive molecule that can interact with a variety of cellular components, it appears likely that NO-induced cytotoxicity can be due to damage to several key cellular constituents which leads to the final demise of the β-cell.

There is a general consensus that DNA is a prime target for many toxic agents because it contains the plans for the cell. Previously, we were able to demonstrate that NO is capable of inducing damage in mitochondrial DNA (mtDNA) at levels that produce significantly less damage to nuclear DNA [10,11]. Damage to these genomes can cause errors in transcription, mutations, recombinations, and rearrangements. Furthermore, mtDNA has no introns and is not protected by histones and, therefore, is more vulnerable to damaging species than nuclear DNA. Thus, its injury would be expected to have a greater impact on cell function, as the probability of damaging coding sequences is much greater. If damage accumulates, electron transport chain function will diminish with a concomitant fall in cell bioenergetics. This would lead to subsequent cellular dysfunction and ultimately cell death. In support of this notion, mtDNA integrity has been shown to be an absolute requirement for normal insulin secretion. Furthermore, our laboratory has shown that the accumulation of mtDNA damage can be one of the key factors triggering apoptosis [12]. This finding has subsequently been corroborated by other investigators [13,14]. Mitochondrial dysfunction due to alterations in the mitochondrial genome has recently attracted much attention, with the finding that mutations in mitochondrially encoded proteins perturb cellular function. Several disorders have been linked to such genetic changes, including a specific diabetic phenotype which may account for 1% of all cases of NIDDM [15]. Because a single mutation can cause a diabetic phenotype when it reaches a certain level of heterogeneity (~60%), we hypothesized that alterations in mtDNA, none of which are present in sufficient quantity to generate a phenotype but collectively rise to a critical level to cause disease, are responsible for other cases of NIDDM. We further hypothesized that NO generated from FFA induction of iNOS is responsible for some of this damage. In an effort to better understand the nature of lipotoxicity induced by FFA, we investigated their capacity to damage mtDNA in INS-1 cells through excessive NO production. The results described herein show that FFA cause a rise in NO that damages mtDNA and ultimately leads to cell death by apoptosis.

Research design and methods

Cell culture

The INS-1 cells used in this study were a gift from Dr. C. Wollheim (Division of Clinical Biochemistry, Department of Internal Medicine, University Medical Center, Switzerland) [16]. The cells were routinely grown at 37°C in RPMI 1640 media supplemented with 10 mM Hepes, 10% fetal bovine serum (Hyclone), 1 mM pyruvate, 50 μM 2-mercaptoethanol, and 50 μg/ml gentamicin sulfate. For experiments, cells were seeded in 100-mm culture dishes 2-3 days prior to the experiments.

Drug preparation and cell exposure

Long-chain free fatty acids (2/1 oleate/palmitate; Sigma) were dissolved in 50% ethanol and used for the treatment of INS-1 cells for various times ranging from 1 to 24 h. BSA at a final concentration of 2% was added to the culture media during FFA treatment. In some experiments with FFA treatment, aminoguanidine (Sigma), an iNOS inhibitor, was added at a concentration of 0.5 mM. For the comparison of DNA damage patterns by ligation-mediated polymerase chain reaction (LM-PCR), INS-1 cells were treated with either 100 μM peroxynitrite (Calbiochem) for 5 min, 100 mM of PAPA/NOate (Cayman Chemical) for 20 min, or 50 mU xanthine oxidase (XO) with 0.5 mM hypoxanthine (both purchased from Sigma) for 20 min.

Assays for mitochondrial DNA damage

INS-1 cells were exposed to FFA or one of the other genotoxins described in the preceding paragraph. Cells were lysed in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.5% SDS, and 0.3 mg/ml proteinase K overnight at 37°C. High molecular weight DNA was extracted with phenol, treated with RNase (to a final concentration of 1 μg/ml), and digested to completion with *Bam*HI overnight. Digested samples were precipitated, resuspended in TE buffer, and precisely quantified using a Hoefer TKO 100 minifluorometer and TKO standard kit. Samples containing 5 μg of DNA were heated for 20 min at 65°C, cooled at room temperature, and incubated with NaOH to a final concentration of 0.1 N for 15 min at 37°C. After alkali treatment, samples were loaded onto a 0.6% alkaline gel and electrophoresed overnight in alkaline buffer consisting of 23 mM NaOH and 1 mM EDTA. After standard gel washing, the DNA was transferred onto a Zeta-Probe GT membrane (Bio-Rad) via vacu-blotting and cross-linked to the membrane. The membrane was hybridized with a ^{32}P-labeled mtDNA specific probe generated via PCR [10]. Hybridization and washes were performed according to the manufacturer's directions. DNA damage was evaluated after densitometry of hybridization bands using Phospho-Analyst software (Bio-Rad).

To evaluate FFA-induced mtDNA damage at the single nucleotide level for comparison with damage induced by known genotoxins, LM-PCR was employed. We have previously published our modification of the LM-PCR technique for the study of mtDNA [17]. Briefly, prior to LM-PCR, aliquots of DNA samples were treated with NaOH to a final concentration of 0.1 N for 15 min at 37°C, precipitated with cold ethanol, and resuspended in TE