Srividya Vasu*, Neville H. McClenaghan, Jane T. McCluskey and Peter R. Flatt

Effects of lipotoxicity on a novel insulin-secreting human pancreatic β-cell line, 1.1B4

Abstract: The novel insulin-secreting human pancreatic β-cell line, 1.1B4, demonstrates stability in culture and many of the secretory functional attributes of human pancreatic β-cells. This study investigated the cellular responses of 1.1B4 cells to lipotoxicity. Chronic 18-h exposure of 1.1B4 cells to 0.5 mM palmitate resulted in decreased cell viability and insulin content. Secretory responses to classical insulinotropic agents and cellular Ca\textsuperscript{2+} handling were also impaired. Palmitate decreased glucokinase activity and mRNA expression of genes involved in secretory function but up-regulated mRNA expression of HSPA5, EIF2A, and EIF2AK3, implicating activation of the endoplasmic reticulum stress response. Palmitate also induced DNA damage and apoptosis of 1.1B4 cells. These responses were accompanied by increased gene expression of the antioxidant enzymes SOD1, SOD2, CAT and GPX1. This study details molecular mechanisms underlying lipotoxicity in 1.1B4 cells and indicates the potential value of the novel β-cell line for future research.

Keywords: 1.1B4; diabetes; mechanisms; palmitate.

*Corresponding author: Srividya Vasu, SAAD Centre for Pharmacy and Diabetes, University of Ulster, Coleraine BT52 1SA, UK, e-mail: vasu-s1@email.ulster.ac.uk

Neville H. McClenaghan, Jane T. McCluskey and Peter R. Flatt: SAAD Centre for Pharmacy and Diabetes, University of Ulster, Coleraine BT52 1SA, UK

Introduction

Increased adiposity due to physical inactivity and obesity leads to elevated free fatty acid levels and cytokines such as interleukin (IL)-1β, interferon (IFN)γ, tumor necrosis factor (TNF)α, IL-6 and the adipokines resistin, leptin and adiponectin (Jonas et al., 2009; McCarthy, 2010). Cytokines and free fatty acids are potentially lethal to pancreatic β cells, while free fatty acids cause insulin resistance (Karaskov et al., 2006; Cvjetičanin et al., 2009). In individuals with insulin resistance, β cells are required to secrete excessive amounts of insulin to lower blood glucose levels. Hence β cells adapt to match insulin demand, which is termed β cell compensation (Bouwens and Rooman, 2005; Del Prato, 2009; Jonas et al., 2009; Sachdeva et al., 2009; Karunakaran et al., 2012). Thus, most individuals with insulin resistance do not develop type 2 diabetes due to β cell hypersecretion until β cell function starts to deteriorate (Weir et al., 2009). Chronic hyperglycemia and chronic hyperlipidemia are extremely toxic to pancreatic β cells (Piro et al., 2002; Cnop et al., 2008; Cunha et al., 2008; Martinez et al., 2008). They result in the induction of apoptosis, which leads to the loss of β cell mass (Bachar et al., 2009; Jonas et al., 2009; Weir et al., 2009).

Mechanisms of lipotoxicity have been studied extensively using rodent pancreatic β-cell models and isolated islets. Proposed mechanisms of lipotoxicity include endoplasmic reticulum (ER) stress and ER stress-induced apoptosis. The ER is well developed in β cells in order to support insulin synthesis and secretory responses to glucose and other agents. Functions of the ER include post-translational modifications, folding and protein trafficking and the provision of cellular Ca\textsuperscript{2+} (Araki et al., 2003). Any disturbances in the normal function would therefore cause β cell stress. Cunha et al. (2008) observed that fatty acids such as palmitate deplete ER Ca\textsuperscript{2+} stores. This causes stress to the ER, which responds to such situations via one or more of three pathways, collectively called the unfolded protein response (UPR) or ER stress response. This includes transcriptional induction of ER chaperones, translational attenuation and degradation of misfolded proteins (Araki et al., 2003). Alleviation of ER stress by UPR results in the resumption of translation. However constant activation of the ER stress response by chronic hyperlipidemia results in the induction of apoptosis by transcriptional induction of CCAAT/enhancer binding protein homologous protein (CHOP) or activation of the c-Jun N terminal Kinase or activation of caspase-12 pathways (Araki et al., 2003).

We have recently developed novel human insulin-secreting cell lines by electrofusion of normal human pancreatic β cells with an immortal cell fusion partner (McCluskey et al., 2011). These cells are stable in culture and exhibit many of the established secretory responses of normal β cells. However nothing is known regarding the suitability of these cells as a model for studies of β cell dysfunction and degeneration. Further knowledge of the molecular and cellular responses of human β cells to
ER stress after exposure to palmitate is far from complete. Hence, the present study has extensively examined the molecular mechanisms underlying the effects of chronic exposure to palmitate on 1.1B4 cell viability, secretory function, gene expression, antioxidant defense, ER stress response and apoptosis.

Results

Viability

Palmitate exerted dose-dependent toxicity on 1.1B4 cells after 18 h’ exposure, with a percentage reduction in metabolic viability of between 25% and 71%, as assessed by MTT assay (p<0.01—<0.001, Figure 1A). The lethal dose (LD₅₀) of palmitate was found to be 0.57 mM, using this assay. As the MTT assay quantifies cell viability based on metabolic activity, the LDH assay was also performed as a more comprehensive method to assess cell viability. Palmitate decreased cell viability by 21–50%, with an LD₅₀ dose of 1.25 mM, as assessed by LDH assay (p<0.001, Figure 1B). As acute exposure to palmitate stimulates β cell secretion rather than dysfunction, a chronic exposure period of 18 h using 0.5 mM palmitate was chosen for investigation of lipotoxicity.

Secretory function

Insulin secretion at 5.6 mM and 16.7 mM glucose was reduced by 44% and 56%, respectively (Figure 2A). KCl, alanine and elevated Ca²⁺ failed to increase insulin output from palmitate-exposed 1.1B4 cells. Similarly glucagon-like peptide-1 and forskolin did not potentiate glucose effects following exposure to 0.5 mM palmitate (p<0.001, Figure 2A). Chronic exposure to palmitate also markedly reduced insulin content (Figure 2B). Activity of the glucose phosphorylating enzyme, glucokinase, was significantly diminished by chronic exposure to palmitate (p<0.01, Figure 2C). Messenger RNA expression of the genes involved in secretory function (INS, GCK, PCSK1, PCSK2 and GJA1) was significantly reduced by palmitate (Table 2). Pancreatic and duodenal homeobox 1 (PDX1) protein expression was significantly down-regulated in palmitate-treated cells (p<0.01, Figure 2D). In addition, stimulatory [Ca²⁺]ᵢ responses to glucose (5.6, 16.7 mM) (Figure 3A), alanine (Figure 3B), arginine (Figure 3B), KCl (Figure 3C) and elevated Ca²⁺ (Figure 3C) were abolished by chronic palmitate exposure. Responses expressed as area-under-the-curve measurements were significantly reduced by 87–95% (p<0.05, p<0.01, and p<0.001, Figure 3D).

Antioxidant defense

Palmitate significantly up-regulated the mRNA expression of SOD1, SOD2, CAT and GPX1, which encode the antioxidant enzymes superoxide dismutase 1, superoxide dismutase 2, catalase and glutathione peroxidase 1, respectively (Table 2).

DNA damage and apoptosis

Chronic palmitate treatment significantly increased olive tail moment and percentage DNA in comet tails, as assessed by comet assay (p<0.001, Figure 4A–D). Induction of apoptosis was evident from a ninefold increase in caspase 3/7 activity in palmitate-treated 1.1B4 cells (p<0.001, Figure 4E). A trend towards a decrease in
Figure 2  (A) Insulin release from 1.1B4 cells after 18 h of exposure to palmitate. Values are mean±SEM (n=8). *p<0.05, **p<0.001 compared to respective control at 5.6 mM glucose. "p<0.01, ""p<0.001 compared to respective secretagogues at untreated control. (B) Insulin content of 1.1B4 cells after 18 h of exposure to palmitate. Values are mean±standard error of the mean (n=8). ***p<0.001 compared to untreated control. (C) Glucokinase enzyme activity after 18 h of exposure to palmitate, expressed in terms of $V_{\text{max}}$ (U/mg), a Michaelis-Menten constant. Values are mean±SEM (n=8). **p<0.01 compared to untreated control. (D) PDX1 protein expression after 18 h of exposure to palmitate. Protein expression was normalized to β-actin expression and plotted as percentage relative band density. Values are mean±SEM (n=3). "p<0.01 compared to untreated control.

BCL2 mRNA level and an increase in BAX mRNA level was observed in palmitate-treated cells (Table 2). The BCL2 protein level was markedly decreased in palmitate-treated cells (p<0.001, Figure 4F). Messenger RNA expression of NFKB1 was markedly increased (p<0.001, Table 2).
Palmitate significantly increased the transcription of HSPA5, which encodes the molecular chaperone BiP, and EIF2A that encodes the eukaryotic translation initiation factor 2α (p<0.01 and p<0.001, Table 2). This was also accompanied by an increase in the transcription of EIF2AK3, which codes for EIF2A kinase (p<0.05, Table 2).

A novel human pancreatic β cell line, 1.1B4 was generated recently by electrofusion of human islet cells and the immortal epithelial cell line PANC-1 (McCluskey et al., 2011). In this study, we investigated the effects of lipotoxicity on 1.1B4 cell viability, secretory function, gene expression and DNA integrity. Exposure to palmitate for 18 h resulted in a concentration-dependent decrease
in cell viability. Similar effects have been observed in RINm5F cells, primary rat, mouse and human β cells, INS-1E cells, MIN6 cells and human islets (Maestre et al., 2003; Cvjetićanin et al., 2009; Hellemans et al., 2009; Choi et al., 2011; Sargsyan and Bergsten, 2011). Lethal dose (LD₅₀) values for palmitate were 576 ± 200 μM for INS-1E cells, MIN6 cells and human islets (Maestre et al., 2003; D’Aleo et al., 2009). Additionally, mRNA expression of GJA1 was down-regulated, suggesting defective communication between neighboring cells to synchronize insulin secretion. As INS gene expression is under the control of the homeodomain transcription factor, PDX1, we investigated whether reduced insulin content was associated with reduced PDX1 levels. Indeed, PDX1 protein expression was very appreciably reduced in palmitate-treated 1.1B4 cells. Similar results were observed in MIN6 cells exposed to palmitate (Martínez et al., 2008). As PDX1 plays a crucial role in the expression of genes involved in the regulation of insulin secretion and maintenance of β-cell mass, a reduction in PDX1 protein could be a major contributor to 1.1B4 cell dysfunction. Thus, secretory defects in 1.1B4 cells caused by palmitate exposure could be mediated via a decrease in PDX1 levels, which leads to a reduction in the expression of β cell-enriched genes including INS, PCSK1, PCSK2 and GCK, resulting in a reduction in insulin content, decrease in glucokinase activity and impairment of Ca²⁺ influx, resulting in defective insulin secretion.

Impairment of secretory function could be secondary to oxidative stress caused by palmitate. Non-esterified fatty acids increase superoxide production in mitochondria, increase the production of NADPH oxidase resulting in increased superoxide levels, and increase the production of hydrogen peroxide in peroxisomes, thus elevating levels of free radicals (Gehrmann et al., 2010). Antioxidant enzymes protect cells from oxidative stress-induced damage by scavenging free radicals. Increased reactive oxygen species levels have been observed in palmitate-treated MIN6 cells, INS-1E cells, isolated mouse and human islets (Maestre et al., 2003; D’Aleo et al., 2009; Saitoh et al., 2010). We observed a substantial up-regulation of SOD1, SOD2, CAT and GPX1 mRNA expression in 1.1B4 cells, suggesting compensatory enhancement of the antioxidant defense mechanisms. Similar effects have been observed previously in human islets and rodent cell lines exposed to palmitate (D’Aleo et al., 2009; Elsner et al., 2011).

Accumulating evidence indicates that lipotoxic effects are also partly mediated by ER stress (Araki et al., 2003; Cunha et al., 2008, 2009). ER stress response or UPR

### Table 2 Gene expression in 1.1B4 cells after exposure to palmitate.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control (%)</th>
<th>18-h palmitate exposure (%)</th>
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<tbody>
<tr>
<td><strong>Secretory function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INS</td>
<td>100±14</td>
<td>38±17</td>
</tr>
<tr>
<td>GCK</td>
<td>100±10</td>
<td>52±5</td>
</tr>
<tr>
<td>PCSK1</td>
<td>100±11</td>
<td>70±14</td>
</tr>
<tr>
<td>PCSK2</td>
<td>100±16</td>
<td>44±5</td>
</tr>
<tr>
<td>GJA1</td>
<td>100±9</td>
<td>67±5</td>
</tr>
<tr>
<td><strong>Antioxidant defense</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD1</td>
<td>100±24</td>
<td>255±15 &quot;&quot;</td>
</tr>
<tr>
<td>SOD2</td>
<td>100±31</td>
<td>255±23 &quot;&quot;</td>
</tr>
<tr>
<td>CAT</td>
<td>100±20</td>
<td>244±25 &quot;&quot;</td>
</tr>
<tr>
<td>GPX1</td>
<td>100±46</td>
<td>470±13 &quot;&quot;</td>
</tr>
<tr>
<td><strong>Apoptosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCL2</td>
<td>100±20</td>
<td>77±15</td>
</tr>
<tr>
<td>BAX</td>
<td>100±17</td>
<td>160±19</td>
</tr>
<tr>
<td>NFKB1</td>
<td>100±13</td>
<td>306±36 &quot;&quot;</td>
</tr>
<tr>
<td><strong>Endoplasmic reticulum stress response</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSPA4</td>
<td>100±16</td>
<td>137±09</td>
</tr>
<tr>
<td>HSPA5</td>
<td>100±34</td>
<td>546±62 &quot;&quot;</td>
</tr>
<tr>
<td>EIF2A</td>
<td>100±13</td>
<td>245±59 &quot;&quot;</td>
</tr>
<tr>
<td>EIF2AK3</td>
<td>100±14</td>
<td>176±13 &quot;&quot;</td>
</tr>
</tbody>
</table>

Following 18 h of exposure to palmitate, mRNA was extracted and converted to cDNA. Real time polymerase chain reaction was carried out and gene expression was normalized to ACTB expression. Values are mean±standard error of the mean (n=3). *p<0.05, **p<0.01, ***p<0.001 compared with respective control (100%).

Thus, the significant reduction of Ca²⁺ entry into 1.1B4 cells could be attributed to changes in the microdomains of Ca²⁺ entry and depletion of ER Ca²⁺ stores.

Palmitate exposure reduced mRNA expression of proinsulin-processing enzymes (PCSK1, PCSK2), GCK and glucokinase enzyme activity in 1.1B4 cells. Reduction in Gck mRNA expression has been reported previously in isolated rat and human islets exposed to palmitate (Yoshikawa et al., 2001; D’Aleo et al., 2009). Additionally, mRNA expression of GJA1 was down-regulated, suggesting defective communication between neighboring cells to synchronize insulin secretion. As INS gene expression is under the control of the homeodomain transcription factor, PDX1, we investigated whether reduced insulin content was associated with reduced PDX1 levels. Indeed, PDX1 protein expression was very appreciably reduced in palmitate-treated 1.1B4 cells. Similar results were observed in MIN6 cells exposed to palmitate (Martínez et al., 2008). As PDX1 plays a crucial role in the expression of genes involved in the regulation of insulin secretion and maintenance of β-cell mass, a reduction in PDX1 protein could be a major contributor to 1.1B4 cell dysfunction. Thus, secretory defects in 1.1B4 cells caused by palmitate exposure could be mediated via a decrease in PDX1 levels, which leads to a reduction in the expression of β cell-enriched genes including INS, PCSK1, PCSK2 and GCK, resulting in a reduction in insulin content, decrease in glucokinase activity and impairment of Ca²⁺ influx, resulting in defective insulin secretion.

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is important for relieving ER stress and protecting cells from apoptosis. Phosphorylation of EIF2A by EIF2AK3 halts protein translation. Several studies have reported an increase in phosphorylated Eif2a and Eif2ak3 levels in palmitate-treated INS-1E cells, Psammomys obesus islets and MIN6 cells (Karaskov et al., 2006; Martinez et al., 2008; Cunha et al., 2008, 2009; Bachar et al., 2009; Gwiazda et al., 2009). We observed an increase in mRNA expression of both EIF2A and EIF2AK3 in 1.1B4 cells, suggesting an increased protein load in the ER after palmitate exposure. Further investigation to assess the phosphorylation status of both EIF2A and EIF2AK3 in 1.1B4 cells could confirm activation of the ER stress response. Up-regulation of the expression of molecular chaperones is a classical feature of ER stress response. Indeed, a marked up-regulation of transcription of the molecular chaperone HSPA5 in treated cells plus a trend towards an up-regulation in HSPA4 transcription was observed. Several other studies have demonstrated up-regulation of molecular chaperones in palmitate-treated INS-1E cells and primary rat β cells (Kharroubi et al., 2004; Cunha et al., 2008; Martinez et al., 2008; Hellemans et al., 2009). This is one of the studies to report up-regulation of molecular chaperone expression in human β cells. Interestingly, NFKB1 transcription was also increased by palmitate and accompanied by up-regulation of molecular chaperone expression and SOD2 transcription. Nuclear factor kappa B (NFKB) activation results in its translocation to the nucleus and the subsequent transcription of influential genes including NOS2, SOD2, CHOP and CHOP4. Further, the NFKB1 gene has a NFKB response element in its promoter, suggesting that it participates in its own regulation (Ten et al., 1992). Thus up-regulation of NFKB1 in 1.1B4 cells after palmitate exposure could be a consequence of NFKB activation.

DNA fragmentation has been observed in INS-1E cells, MIN6 cells and human islets exposed to palmitate (Maestre et al., 2003; Choi et al., 2011; Lee et al., 2011; Sargsyan and Bergsten, 2011). The fact that 1.1B4 DNA integrity was compromised by palmitate treatment was evident from a marked increase in the percentage tail DNA in comet tails. Olive tail moment, which is a direct measure of the extent of...
DNA damage, was 15-fold higher in palmitate-treated cells. Excessive DNA fragmentation, which is induced by reactive oxygen species generation, results in the induction of apoptosis. Caspase 3, an effector molecule of apoptosis, is activated by its cleavage into 17 and 12 kDa subunits. Cleaved caspase 3 level or caspase 3 activity was higher in palmitate-treated RINm5F cells, INS-1E cells, MIN6 cells and human islets (Rakatzi et al., 2004; Martinez et al., 2008; Bachar et al., 2009; Cunha et al., 2009; Cvetičanin et al., 2009; Chu et al., 2010; Choi et al., 2011; Lee et al., 2011; Sargsyan and Bergsten, 2011). In 1.1B4 cells exposed to palmitate, caspase 3/7 activity was increased by ninefold, suggesting the execution of apoptosis. In addition, BCL2 transcription was reduced, with a marked reduction in BCL2 protein levels, while BAX transcription was increased in palmitate-treated 1.1B4 cells, which has been observed in INS-1E cells and isolated human islets exposed to palmitate (Cunha et al., 2009; D’Aleo et al., 2009).

In conclusion, this first study on the cellular and molecular responses of novel human 1.1B4 cells to palmitate emphasizes the potential of this model for research on destructive mechanisms in human β cells. Such investigations could aid a better understanding of the pathogenesis of diabetes and shed light on undiscovered pathways that may be harnessed for the development of therapeutic strategies.

Materials and methods

Cell culture and viability

The 1.1B4 cells were routinely cultured in RPMI-1640 medium (Gibco®, Invitrogen, UK) containing 11.1 mM glucose, 10% (v/v) fetal bovine serum (Gibco®, Invitrogen, UK) and 1% (v/v) antibiotics [penicillin (100 U/ml) and streptomycin (0.1 mg/l)] (Gibco®,
Insulin release, insulin content and glucokinase

The 1.1B4 cells were harvested and seeded at a density of 70,000 cells per well in 24-well plates and allowed to attach overnight. Following 18 h of palmitate treatment, acute insulin release studies were performed as previously described (McCluskey et al., 2011). To assess the secretory responsiveness of 1.1B4 cells, insulin secretion after 20 min of exposure to a range of secretagogues acting at different points in signal transduction including glucose (5.6, 16.7 mM), alanine (10 mM), KCl (30 mM), elevated Ca²⁺ (7.68 mM), glucagon-like peptide-1 (2 μM) and forskolin (10 μM) was assessed. For analysis of insulin content, cells were extracted using ice-cold acid ethanol (75% v/v ethanol, 1.5% v/v concentrated HCl). Insulin was measured by radioimmunoassay using human insulin standards (Flatt and Bailey, 1981). To assess changes at the level of β-cell glucose sensing, glucose phosphorylating enzyme activity was determined. Total protein was extracted from 1.1B4 cells by sonication and subjected to an enzyme-coupled photometric assay (Lenzen et al., 1987). Glucokinase activity was measured by subtracting the hexokinase activity at 1 mM/glucose from total activity at 100 mM/glucose.

Intracellular Ca²⁺ studies

The [Ca²⁺], responses were determined using FLIPR Calcium 5 assay kit (Molecular Devices, Sunnyvale, CA, USA) following the manufacturer’s instructions. The 1.1B4 cells were seeded into 96-well, black-walled, clear-bottom plates at a density of 25,000 cells per well and allowed to attach overnight. Intracellular Ca²⁺ responses were monitored using a FlexStation scanning fluorometer (Molecular Devices, Sunnyvale, CA, USA) with integrated fluid transfer work station (Miguel et al., 2004).

Real-time reverse transcription polymerase chain reaction

Messenger RNA was extracted from 1.1B4 cells using RNeasy mini kit (Qiagen, Manchester, UK) according to the manufacturer’s instructions. One to 5 μg of mRNA was converted to cDNA using Superscript II reverse transcriptase RNase H kit (Invitrogen, Paisley, UK). A Quantifast SYBR green polymerase chain reaction (PCR) kit (Qiagen, Manchester, UK) was used for real-time reverse transcription PCR, with a reaction mix containing 12.5 μl PCR master mix, 1 μl primers (forward and reverse, Invitrogen, Paisley, UK), 1 μl cDNA and 9.5 μl RNase-free water. Amplification conditions were initial denaturation at 95°C for 5 min, final denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, with melting curve analysis at a temperature range of 60–90°C. Each PCR experiment included a negative control and a positive control (ACTB). MiniOpticon two-color real time PCR detection system (BioRad, Hertfordshire, UK) was used for data acquisition. Results were analyzed using the ΔΔ Ct method, with mRNA expression normalized to ACTB. Only those primers with efficiencies between 90 and 110% were used (Table 1).

Protein extraction, quantification and Western blotting

The 1.1B4 cells were harvested and total protein was extracted using an extraction buffer containing 150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 50 mM Tris HCl, pH 7.6, with protease inhibitor cocktail at 4°C for 10 min. The relative protein expression of BCL2 and PDX1 was determined by Western blot analysis. Each sample, containing 30 μg protein, was boiled at 95°C with Laemmli buffer for 10 min, loaded on to pre-cast gels (NUPAGE 4–12% Bis-Tris gels, Invitrogen, Paisley, UK) and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (100 V, 45 min). Following transfer, membranes were blocked with 5% non-fat dried milk and probed with rabbit polyclonal antibody to Bcl2 (1:200)/rabbit polyclonal antibody to PDX1 (1:5000)/mouse monoclonal antibody to β-actin (1:5000) (Abcam, Cambridge, UK). The membranes were then probed with ECL anti-rabbit immunoglobulin G horseradish peroxidase-linked whole antibody (from donkey)/ECL anti-mouse immunoglobulin G horseradish peroxidase-linked whole antibody (from sheep) (1:10000) (GE Healthcare, Amersham, UK) and detected using Luminata Forte HRP substrate (Millipore, Watford, UK).

Alkaline comet assay

The 1.1B4 cells were harvested and an alkaline comet assay was performed to determine the DNA damage (Lees Murdock et al., 2004). For positive control, healthy cells were treated with ultraviolet light for 30 min. After electrophoresis, the cells were stained with 4′,6-diamidino-2-phenylindole (100 μg/ml) and viewed using epifluorescence microscope (Olympus system microscope, model BX51). The DNA damage was assessed by CometScore software, which analyses complete comets measuring densitometric and geometric parameters including the percentage tail DNA and olive tail moment. Four replicates per treatment and 100 cells per gel were analyzed.

Caspase assay

Caspase assay was carried out using Caspase Glo 3/7 assay kit (Promega, Southampton, UK) according to the manufacturer’s instructions. Briefly, 1.1B4 cells were lysed using 1 ml of cold RIPA buffer (without the addition of protease inhibitor to protect caspase activity) and extracted protein samples were analyzed for caspase 3/7 activity. Luminescence was measured using Flexstation III (Molecular Devices, Sunnyvale, CA, USA). Caspase 3/7 activity was expressed in terms of relative luminescence units.
Statistical analysis

Results were analyzed in GraphPad PRISM (version 3.0) and presented as mean±standard error of the mean. Statistical analyses were carried out by unpaired student’s t-test (non-parametric) (with two-tailed p-values and 95% confidence intervals). The results were considered significant if they were p<0.05.

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