EXPERIMENTAL CELL RESEARCH 319 (2013) 3035–3041

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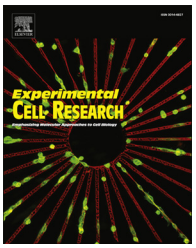

Research Article

GW9508, a free fatty acid receptor agonist, specifically induces cell death in bone resorbing precursor cells through increased oxidative stress from mitochondrial origin

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ARTICLE INFORMATION

Article Chronology:
Received 8 April 2013
Received in revised form
6 August 2013
Accepted 7 August 2013
Available online 22 August 2013

Keywords:
Free fatty acid receptor agonist
Bone
Cell death
Oxidative stress

ABSTRACT

GW9508 is a free fatty acid receptor agonist able to protect from ovariectomy-induced bone loss *in vivo* thought inhibition of osteoclast differentiation in a G-coupled Protein Receptor 40 (GPR40)-dependent way. In this study, we questioned whether higher doses of GW9508 may also influence resorbing cell viability specifically. Interestingly, GW9508 at 100 μM altered osteoclast precursor (OcP) viability while it had positive effects on osteoblastic precursors suggesting an activity dependent on the cell lineage. According to 7-AAD/Annexin-V staining, induced OcP cell death was found to be associated with necrosis mechanisms. Consistently, GW9508 led to a sustained establishment of oxidative stress from mitochondrial origin. In contrast to previous observations on osteoclast differentiation inhibition, OcP viability targeted by high doses of GW9508 appeared to be independent of GPR40 involvement. Although mediating structures remain to be determined, our data demonstrate for the first time that this fatty acid receptor agonist driving OcP specific cell death may now open new perspectives regarding therapeutic strategies in osteolytic disorders.

Introduction

Recently, orphan membrane G- protein coupled receptors (GPRCs) such as GPR40, 41, 43 and 120 [1] have been identified as fatty acid receptors. GPR41 and 43 are short chain fatty acid receptors while GPR40 and GPR120 preferentially bind medium/long chain fatty acids. GPR40 has mainly been studied in β-pancreatic cell functions for its role in potentiating glucose dependent insulin secretion in the presence of free fatty acids [2–5]. Latterly, GPR40 functions were extended to brain tissues for its role in adult neurogenesis and memory mechanisms [6]. More generally, GPR40 play a pivotal role in nutrisensing along the gastro-intestinal tract, most notably for mediating fat related taste in tong [7] and fat sensing in the intestine [8]. On the other hand, GPR120 was reported to mediate the anti-inflammatory effects of ω-3 polyunsaturated fatty acids (PUFA) in macrophages [9].

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http://dx.doi.org/10.1016/j.yexcr.2013.08.013

Regarding mineralized tissues, we and others previously demonstrated that GPR40 is also expressed in bone cells [10,11] and that GPR40−/− mice exhibit osteoporotic features [12]. This phenotype relies on a GPR40 protective effect towards bone matrix. Indeed, *in vivo*, GPR40 stimulation protects from bone loss in an ovariectomized mouse model through inhibition of osteoclast differentiation. *In vitro*, we showed that doses as low as 50 μM of the GPR40 agonist GW9508 targeted NF-κB pathway by blocking its transcriptional activity and subsequent osteoclast differentiation without affecting cell viability [13].

However, some of these fatty acid receptors were recently shown to differently impact cell behavior from several origins. GPR43 was proven to induce caspase activation and increase apoptotic cell death upon propionate/butyrate treatment in HCT8 human colonic adenocarcinoma cells [14]. Along with GPR43, Wu et al. demonstrated that GPR40 promote palmitate-induced ER stress and apoptosis in MIN6 β cells [15]. In contrast, GPR120 activation was found to inhibit serum deprivation-induced apoptosis in a murine enteroendocrine cell line STC-1 [16].

In the light of these seemingly conflicting data, and regarding the impact of pathological bone resorption in different health conditions, we questioned whether higher doses of GW9508 may influence resorbing cell viability specifically and investigated occurring mechanisms. In this study we demonstrate that high doses of GW9508 lead to an increase in pre-osteoclast (OcP) cell death through increased oxidative stress in a cell origin dependent manner.

Thus, according to our results, this fatty acid receptor agonist driving OcP specific cell death could lead to new opportunities in therapeutic strategies specifically targeting osteolytic disorders including periprosthetic and tumors associated bone loss.

Materials and methods

Cell lines

The murine osteoclast and osteoblast precursors cell lines RAW 264.7 and MC3T3-E1 were obtained from the American Type Culture Collection (ATCC Numbers: TIB-71 and CRL-2593 respectively). For all experiments, cells were seeded at a density of 3×10^4 cells/cm² and grown in α-MEM medium without phenol red supplemented with 10% FCS. At 80% confluency, cells were serum starved for a total period of 48 hours for each condition including GPR40 agonist incubation periods.

Cytotoxicity assay (XTT)

Cell cytotoxicity was determined by the XTT based method, using the Cell Proliferation Kit II (Roche) according to the supplier′s recommendations. As described above, cells were first serum starved and then incubated for 24 h in the presence of vehicule (DMSO) or 1–100 μM of GW9508 (Cayman Chemical–CAS 885101-89-3). Optical density was measured on a microplate reader using an absorbance wavelength of 450 nm, with a reference wavelength of 650 nm.

Cell death: Annexin V/7-Amino-Actinomycin (7-AAD) staining

RAW 264.7 were cultured in the presence of vehicule (DMSO) or 100 μM of GW9508 for increasing period of time ranging from 3 to 24 h prior cell staining. Staining was analyzed by fluorescent activated cell sorting using the PE Annexin V Apoptosis Detection Kit I (BD Pharmingen) according to the supplier′s recommendations. Annexin V staining was detected by phycoerythrin (PE) emission signal detector (FL2) and 7-AAD staining by FL3. 10,000 positive events were used to estimate the distribution within the total population. Figures show representative data from three independent experiments.

Assay of caspase-3 activity

The CaspACE™ assay system (Promega) was used to quantify caspase-3 activity in cell lysates following the manufacturer′s protocol. Briefly, after 24 h of incubation with GW9508, RAW264.7 were washed with ice-cold PBS and lysed in ice-cold lysis buffer (NaCl 150 mM, Tris 50 mM, Nonidet P-40 1%, sodium deoxycholate 0.25%, NaF 1 mM, NaVO4 1 mM, leupeptine 10 μg/ml, aprotinin 10 μg/ml, PMSF 0.5 mM). For each treatment group, an equal amount of soluble protein was incubated with 50 μM acetyl-Asp-Glu-Val-Asp 7-amino-4-methyl coumarin (Promega), a fluorogenic substrate for caspase-3, with or without 50 μM acetyl-Asp-Glu-Val-Asp aldehyde, a specific caspase-3 inhibitor (Promega). Cell lysates were pre-incubated with the inhibitor for 30 min prior to adding the substrate and cleavage of the substrate was analyzed using a fluorometer excitation wavelength 360 nm, detection wavelength 460 nm.

Measurement of reactive oxygen species (ROS) production (DCF-DA)

Intracellular reactive oxygen species (ROS) were detected using 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) (Invitrogen). RAW 264.7 were cultured for 1, 2, 3 or 24 h either in the presence of vehicule (DMSO) or 100 μM of GW9508. Cells were washed twice with warm PBS and incubated for 30 min at 37 °C (in the dark) with the probe at a final concentration of 2.5 μM dye. Cells were washed twice with PBS and harvested. The fluorescence intensity was quantified by flow cytometry (FL1). 10,000 positive events were used to estimate the distribution within the total population. Figures show representative data from three independent experiments.

Measurement of mitochondrial ROS production (Mitosox)

Intracellular mitochondrial reactive oxygen species (ROS) production were detected using MitoSOX Red mitochondrial superoxide indicator for live-cell imaging (Invitrogen) according to the supplier′s recommendations. RAW 264.7 were cultured for 24 h in the presence of vehicule (DMSO) or 100 μM of GW9508. The fluorescence intensity was quantified by flow cytometry (FL1). 10,000 positive events were used to estimate the distribution within the total population. Figures show representative data from three independent experiments.

Measurement of Mitochondrial Membrane Potential (JC-1)

Mitochondrial membrane potential (MMP) was estimated using 5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Invitrogen). As described previously, RAW 264.7 were cultured for 24 h in the presence of vehicule (DMSO)