Glyphosate Inhibits PPAR Gamma Induction and Differentiation of Preadipocytes and is able to Induce Oxidative Stress

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ABSTRACT: Glyphosate-based herbicides (GF) are extensively used for weed control. Thus, it is important to investigate their putative toxic effects. We have reported that GF at subagriculture concentrations inhibits proliferation and differentiation to adipocytes of 3T3-L1 fibroblasts. In this investigation, we evaluated the effect of GF on genes upregulated during adipogenesis. GF was able to inhibit the induction of PPAR gamma, the master gene in adipogenesis but not C/EBP beta, which precedes PPAR gamma activation. GF also inhibited differentiation and proliferation of another model of preadipocyte: mouse embryonic fibroblasts. In exponentially growing 3T3-L1 cells, GF increased lipid peroxidation and the activity of the antioxidant enzyme, superoxide dismutase. We also found that proliferation was inhibited with lower concentrations of GF when time of exposure was extended. Thus, GF was able to inhibit proliferation and differentiation of preadipocytes and to induce oxidative stress, which is indicative of its ability to alter cellular physiology.
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KEYWORDS: **Differentiation; Glyphosate Based-Herbicide; Oxidative Stress; 3T3-L1 Fibroblasts; MEFs**

INTRODUCTION

Glyphosate is the active ingredient of the most commonly used commercial herbicide worldwide and its effect is due to the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, which is involved in the synthesis of several essential amino acids in plants but it is not found in animals [1, 2]. Thus, glyphosate is considered to be safe to people and other vertebrates when used according to the manufacturer's instructions.

Glyphosate-based herbicides are extensively used in countries such as US, Argentina, Brazil, and Canada, which have large plantings of glyphosate-resistant crops. In Argentina, glyphosate was found in water and soil of an area of Buenos Aires planted with genetically modified soybean [3]. Taking this into account, it is possible for this herbicide to spread in the ecosystem and reach plants, animals, and also the food chain. In addition, humans may be exposed to herbicide residues by agriculture practices [4].

A commercial formulation of glyphosate, Roundup®, has been reported to be genotoxic [5] and to have teratogenic effects in *Xenopus laevis* and chicken embryos [6]. It has also been shown to inhibit cell cycle progression from analysis of the first cell division of sea urchin embryos, a recognized model for cell cycle studies [7].

Glyphosate-based herbicides also contain adjuvants, such as polyoxyethyleneamine, which facilitate the absorption of the herbicide. These compounds are considered as inert but may also be toxic. In fact commercial formulations of glyphosate have been shown to be more toxic and more potent endocrine disruptors than the active ingredient itself [8–10]. Thus, the requirement of testing the toxic effects of their whole formulations as mixtures and not only the active principles has been indicated [10, 11].

3T3-L1 fibroblasts are a useful tool in the study of adipocyte differentiation. After the addition of a differentiation mixture (DM) containing insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (MIX),

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two early transcription factors CCAT enhancer-binding protein beta (C/EBP beta) and C/EBP delta are induced during the first hours and then they trigger the expression of a key transcription factor in adipogenesis: peroxisome proliferator activated receptor gamma (PPAR gamma), which is involved in the induction of proteins associated with adipocyte phenotype [12].

3T3-L1 fibroblasts were previously used to evaluate the role of arsenic trioxide, as well as hexavalent chromium on cell death and differentiation [13, 14]. Therefore, 3T3-L1 fibroblasts are an interesting mammalian cellular model that allows to evaluate the effect of environmental contaminants on two different physiological processes that take place in these cells: proliferation and differentiation.

We have previously shown that a commercial glyphosate formulation (GF) inhibits proliferation in 3T3-L1 fibroblasts and induces apoptosis, which is indicative of cellular damage and also inhibits the ability of this cell line to differentiate to adipocytes [15] . In the present investigation, we evaluated the effect of GF on genes necessary for differentiation of 3T3-L1 to adipocytes as well as its effect on proliferation and differentiation of an extensively used model of primary culture of preadipocytes, mouse embryonic fibroblasts (MEFs). In addition, GF-mediated induction of oxidative stress was also evaluated.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle medium (DMEM), trypsin, insulin, Bodipy 493/503, and Alexa Fluor 568-conjugated secondary antibody were obtained from Invitrogen (Carlsbad, CA); MIX dexamethasone, Hoechst 33258, Oil-Red-O, and pure glyphosate were purchased from Sigma Chemical Co. (St. Louis, MO). Glifosato Atanor (48% w/v, isopropylamine salt, 35.6% w/v acid equivalent [ae]), this formulation was from Atanor, Argentina. According to the supplier, this herbicide contains inerts and water but no specification is provided for commercial reasons.

Cell Cultures and Treatments

3T3-L1 fibroblasts were obtained from Asociación Banco Argentino de Células (origin: ATCC). These fibroblasts were cultured in DMEM + 10% fetal bovine serum (FBS) with 100 μg/mL streptomycin, 100 U/mL penicillin, and 250 ng/mL fungizone (DMEM + 10% FBS). When indicated, GF and pure glyphosate (GLY) were added in DMEM + 10% FBS as vehicle. Prior to addition of GF or GLY to the cell plate, the appropriate dilution was prepared and neutralized with a small amount of NaOH, when it was necessary to keep the pH of the medium, since GF and GLY are acid.

MEFs were prepared from CF-1 mouse embryos at day 14 of gestation. After removing head, limbs, and red organs, embryos were finely minced, digested with 0.25% trypsin-1 mM EDTA for 30 min at 37°C. Then, the cell suspension was pipetted up and down, diluted with culture medium and finally centrifuged for 5 min at 1000 $\times$ g . The pellet was resuspended in culture medium before plating. Cells were cultured at 37°C in DMEM with 10% FBS similarly as it was described for 3T3-L1 fibroblasts. For experiments, MEFs from passages 2 to 4 were used. The protocol for the research project was approved by the Ethics Committee of the School of Sciences at the University of Buenos Aires and it conforms to the provisions of the Declaration of Helsinki.

Cell Counting in Exponentially Growing Cells

MEFs were cultured in 24-well plates until they reached 20%–30% confluence. At that moment, some wells were treated with different doses of GF for 24 h and others were treated with DMEM + 10% FBS alone (control). At the end of these treatments, cells were trypsinized, resuspended in PBS and an aliquot was counted using a Neubauer chamber.

Induction of Differentiation

To induce differentiation, 2-days post-confluent cells were treated with a DM containing 10 μg/mL insulin, 0.5 mM MIX, and 100 nM dexamethasone in DMEM + 10% FBS. Three days after the induction of differentiation, medium was replaced with DMEM + 10% FBS supplemented with 10 μg/mL insulin. Then, medium was changed every 2 days with DMEM + 10% FBS. When indicated, GF or GLY was added, as described in each experiment, to obtain the appropriate final concentration.

Oil- Red-O Staining

To visualize cytosolic lipids, Oil-Red-O staining was used [16]. Seven days after induction of differentiation, adipocyte monolayers were washed three times with PBS and then fixed for 30 min with 4% formaldehyde in PBS. Oil-Red-O (0.35%) in isopropanol was diluted with water (3:2), filtered, and added to the fixed cell monolayers for 30 min at room temperature. Cells were then washed with water and the stained

triglyceride droplets in the cells were visualized and photographed. Microphotographies were taken with an Olympus IX-71 inverted microscope equipped with a QImaging EXiAQUA camera. To measure the content of lipids, Oil-Red-O-stained lipids were dissolved in isopropanol and then quantified by measuring the absorbance at 492 nm [16]

RNA Extraction and RT-qPCR Analysis

3T3-L1 fibroblasts or MEFs were cultured in 12-well plates and 3 or 7 days after addition of DM in the absence (DM) or presence of GF (DM+GF) or of medium alone (C), cells were lysed in TRI Reagent (Molecular Research Center Cincinnati, OH, USA). RNA extraction was performed according to the manufacturer's instructions and pellets were resuspended in nuclease-free water. RNA was quantified with NanoDrop 2000 spectrophotometer (Thermo Scientific Waltham, MA USA), and 2 μg were used for reverse transcription using MMLV Reverse Transcriptase (Promega Madison, Wi, USA) and oligo dT_{15}. Relative expression of cDNAs was determined by quantitative real-time PCR using a Bio-Rad MyIQ2 thermal cycler. Each PCR reaction was performed in a final volume of 25 μL containing 5 μL of a 1:10 dilution of first-strand cDNA, 15 pmol of each primer, 0.8 mM dNTPs, 3 mM $MgCl_2$, 0.5 U of Platinum Taq DNA Polymerase (Invitrogen) and SYBR Green (Invitrogen Carlsbad, Ca, USA) in a buffer supplied by the manufacturer. All reactions were performed using the following cycling conditions: 94°C for 5 min, followed by 40 cycles of 94°C for 20 s, 58°C for 30 s and 72°C for 40 s. Target gene mRNA expression was normalized to acidic ribosomal protein (Rplp0) as a reference gene. The following primers were used: PPAR gamma 2, Fwd: CCAGAGCATGGTGCCTTCGCT, Rev: CAGCAACCATTGGGTCAGCTC; and Rplp0, Fwd: GAGGAATCAGATGAGGATATGGGA, Rev: AAGCAGGCTGACTTGGTTGC.

Western-Blot Determination

Two days post-confluent 3T3-L1 fibroblasts were induced to differentiate with DM in the absence (DM) or presence of GF at 1:2000 dilution (DM + GF) or were treated with medium alone (C). After 1 day for C/EBP beta or 7 days for PPAR gamma, cells were harvested in PBS, vortexed and before the addition of Laemmli sample buffer [17] an aliquot was obtained for protein determination using the method of Bradford with serum albumin as standard [18]. After SDS–PAGE using equal amounts of protein in each lane (5–30 μg), gels were transferred to nitrocellulose membrane, blocked with 5% milk for an hour and then treated overnight with the corresponding antibodies (anti-C/EBP beta [C-19] from Santa Cruz or PPAR gamma [C26H12] from Cell Signaling Danvers, MA,USA) followed by treatment with HRP-conjugated secondary antibody (from Santa Cruz) for 1 h. Finally, chemiluminiscence reagent (from Sigma) was used for detection. GAPDH (anti-GAPDH [6C5] from Santa Cruz, CA, USA) was used as loading control.

Immunofluorescence Microscopy for PPAR Gamma and Lipid Detection

Fibroblasts were cultured in 24-well plates and 4 days after induction of differentiation, in the absence (DM) or presence of GF 1:2000 (DM+GF) or medium alone (C), cells were fixed in 4% formaldehyde, permeabilized in PBS-0.25% Triton X-100 and blocked with 5% bovine serum albumin. Then cells were incubated with anti-PPAR gamma antibody ([C26H12] from Cell Signaling) followed by Alexa 568-conjugated secondary antibody. Finally the cells were stained with Bodipy 493/503 for visualization of the lipid droplets, and with Hoechst 33258 for visualization of the nuclei. Cells were visualized and photographed in an Olympus IX-71 inverted microscope equipped with a QImaging EXiAQUA camera.

Quantification of Lipid Peroxidation Based on Fe(III)–Xylenol Orange Complex Formation

A modification of a method previously described [19] was used. 3T3-L1 fibroblasts were cultured in SIX-well plates until they reached 50% confluence. At this time, cells were treated for 24 h with medium (C) or with GF final dil 1:2000 (GF 1:2000). After the treatment, cells were trypsinized, washed twice with PBS and resuspended in PBS. An aliquot was separated for protein determination using the method of Bradford with serum albumin as standard [18]. Then cells were centrifuged, the supernatants were removed and the pellets of cells were resuspended in methanol. The methanolic extracts were centrifuged and the supernatants were used for the assay. The following reagents were added sequentially: 0.25 mM $FeSO_4$, 25 mM H_2SO_4, 0.1 mM xylenol orange and water to complete a total volume of 0.9 ml and then 0.1 mL of each supernatant was added. A blank with 0.1 mL of water was prepared. Samples were incubated at room temperature for 2 h and absorbance at 580 nm was then determined. Units are calculated from relative absorbance and values are given as Units/μg protein.

Determination of Superoxide Dismutase Activity

3T3-L1 fibroblasts were cultured in six-well plates until they reached 50% confluence. At this time, cells were treated for 24 h with medium (C) or with GF final dil 1:2000 (GF 1:2000). After these treatments, cells were lysed in 250 μL de PBS and transferred to eppendorf tubes. An aliquot was separated for protein determination using the method of Bradford [18] and another aliquot was used to measure superoxide dismutase activity (SOD) with RANSOD kit (RANDOX Lab., Crumlin, Antrim, U.K.). Briefly, 1.7 mL of the mixed substrate (50 μM Xanthine and 25 μM INT (2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride)) and 250 μL of Xantine Oxidase (80 U/L) were added to 50 μL of cell lysate. Absorbance at 505 nm was determined at 30 s and then at 3 min. SOD units in the sample were calculated from the difference between the two absorbance values using a calibration curve.

Statistical Analysis

The experiments were carried out three times unless otherwise stated. All data are expressed as mean $\pm$ S.E. Statistical analysis was performed by One-Way ANOVA followed by Tukey's posthoc test or, when indicated, Student´s *t*-test was used. *p* Values below 0.05 were considered significant.

RESULTS

Effect of Glyphosate Formulation on the Amount of PPAR Gamma and C/EBP Beta During Differentiation of 3T3-L1 Fibroblasts to Adipocytes

In a previous work [15], we have reported that a GF at subagriculture concentrations is able to inhibit the proliferation of 3T3-L1 fibroblasts, as well as, the accumulation of cytosolic lipids that takes place when these cells are induced to differentiate to adipocytes. The inhibition of adipogenesis by 1:2000 dilution of GF is shown in Figure 1A where a significant decrease in Oil-red-O-stained lipids was seen after this treatment. This was confirmed by the quantification of stained lipids included in Figure 1A. Taking into account that PPAR gamma is upregulated in adipogenesis and is the master gene in this differentiation process [12], we wanted to investigate the effect of GF treatment on the expression of this transcription factor. We first quantified PPAR gamma mRNA by RT-qPCR and found an increase of this mRNA in cells treated with DM with respect to undifferentiated 3T3-L1 fibroblasts, in agreement with the upregulation of this transcription factor in adipogenesis. The induction of PPAR gamma was inhibited when GF was present during differentiation of 3T3-L1 fibroblasts (Figure 1B), which is in keeping with the ability of GF to inhibit differentiation to adipocytes [15].

On the other hand, we found that pure glyphosate (GLY) did not significantly inhibit differentiation of 3T3-L1 fibroblasts or PPAR gamma induction at a concentration similar to that present in a 1:2000 dilution of GF, which corresponds to 178 ppm of ae, although inhibition was found at a higher concentration of glyphosate alone (equivalent to that found in a 1:100 dilution of GF, i.e., 3600 ppm ae) (Figures 1A and 1B), which is in agreement with previous findings that highlight the contribution of adjuvants present in commercial formulations. Our results further prove the importance of testing the cytotoxic ability of whole formulations rather than glyphosate alone as was proposed by others [8, 10, 11], since these formulations are actually used in agriculture practices. Taking this into account, we continued studying GF effects.

The effects of GF on PPAR gamma were confirmed by quantification of the amount of this protein by Western blot. As expected, this protein was increased in samples treated with DM with respect to control cells. However, when cells were treated with DM in the presence of GF, this increase was inhibited (Figure 1C).

To further prove this effect of the GF, we analyzed nuclear PPAR gamma content by immunofluorescence and cytosolic lipid accumulation by Bodipy 493/503 staining in cells differentiated in the presence or absence of GF for four days. At this time of the differentiation process, PPAR gamma is known to be increased but cytosolic lipid droplets are beginning to appear. As shown in Figure 2, GF inhibited the increases found in PPAR gamma and cytosolic lipids four days after induction of differentiation.

The effect of GF on the amount of an early activated transcription factor in adipogenesis, C/EBP beta, which is known to upregulate PPAR gamma, was also investigated. As expected, the amount of C/EBP beta evaluated by Western blot, was increased 24 h after the induction of differentiation with respect to undifferentiated cells. The increase in C/EBP beta was not inhibited when differentiation was carried out in the presence of GF (Figure 1D).

Effect of Glyphosate Formulation on the Proliferation of MEFs

To confirm the ability of glyphosate formulation to inhibit proliferation and differentiation of preadipocytes, as well as its effect on PPAR gamma

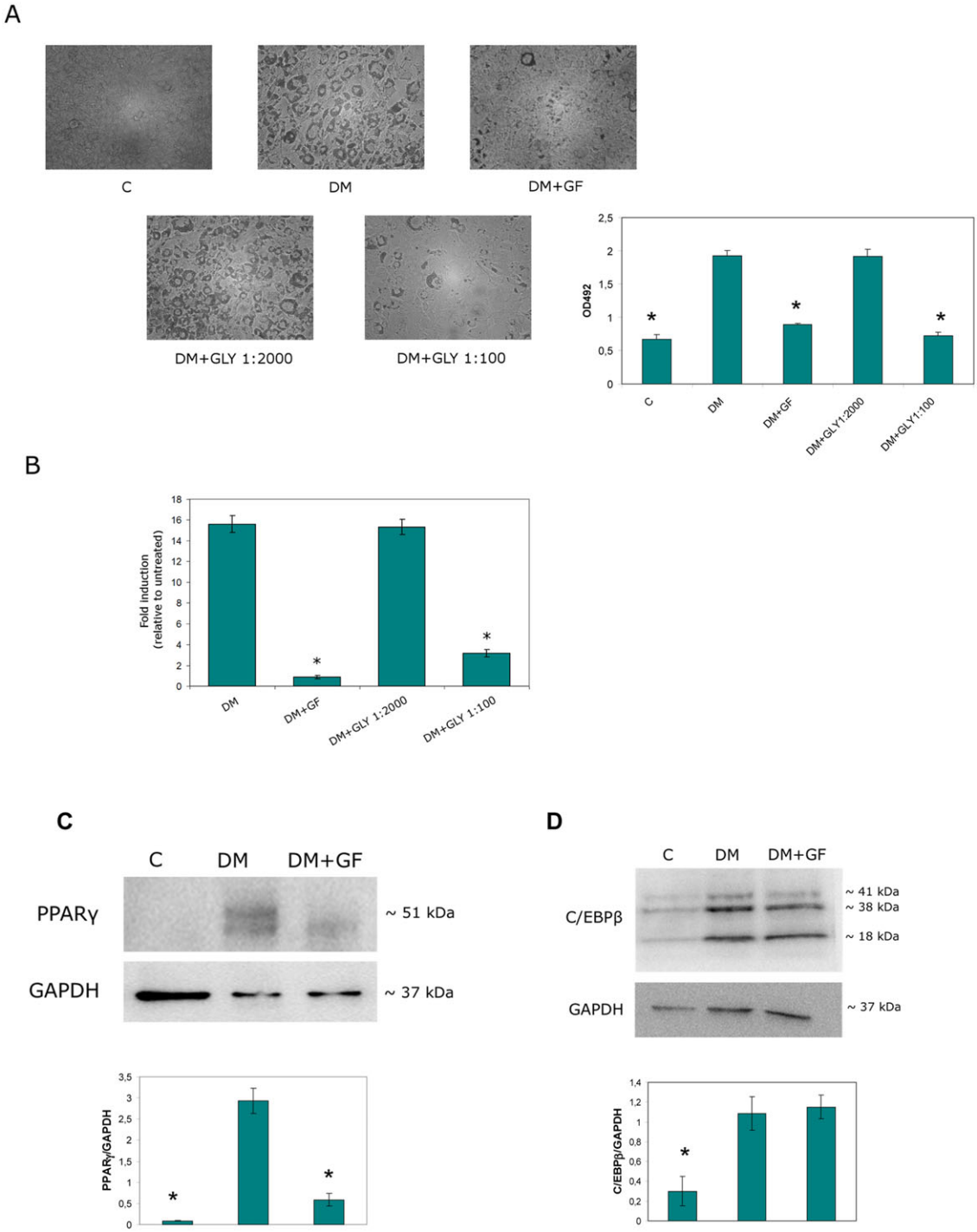

FIGURE 1. Effect of GF on the differentiation of 3T3-L1 fibroblasts. Two days post-confluent cells were treated with: medium alone (C), or with the addition of differentiation mixture (DM), or differentiation mixture plus 1:2000 dilution of GF (DM+GF), or plus two different concentrations of glyphosate equivalent to a 1:2000 and 1:100 dilution of GF (DM+GLY 1:2000 and DM+GLY 1:100, respectively). GF and GLY were maintained in the medium supplemented with insulin that was added 3 days after induction of differentiation. Seven days after induction of differentiation, adipocytes were (**A**) stained with Oil-Red-O and photographed. Lipid accumulation was evaluated by determination of OD 492 of Oil-Red-O-stained lipids as described in section *Materials and Methods* or (**B**) were used to analyze the amount of PPAR gamma mRNA by RT-qPCR as indicated in section *Materials and Methods*. RT-qPCR data are expressed relative to untreated cells that is set to 1 and represent mean ± S.E. Results are from a representative experiment repeated three times with similar results. * Significantly different from DM, $p < 0.05$ (ANOVA, Tukey's test). (**C**) The amount of PPAR gamma was analyzed after 7 days and (**D**) C/EBP beta was analyzed after 24 h of induction of differentiation using Western blot as indicated in section *Materials and Methods*. Densitometric analysis of the Western blots are shown. Results are from a representative experiment repeated three times with similar results. * Significantly different from DM, $p < 0.05$ (ANOVA, Tukey's test).

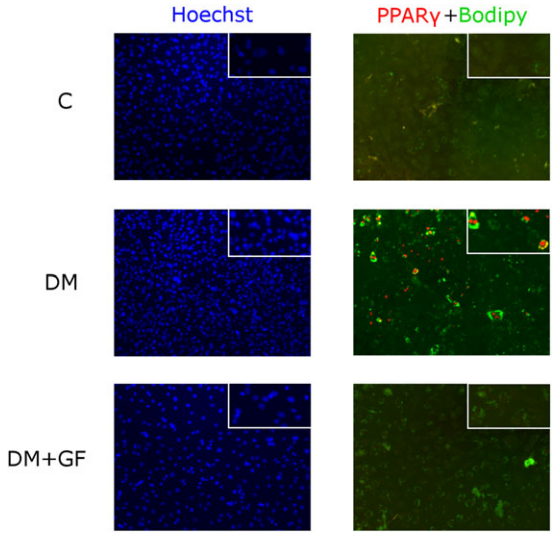

FIGURE 2. Effect of GF on nuclear PPAR gamma during differentiation of 3T3-L1 fibroblasts. Two days post-confluent cells were treated with: medium alone (C), or with the addition of differentiation mixture (DM), or differentiation mixture plus 1:2000 dilution of GF (DM+GF). At day 4, the amount of nuclear PPAR gamma was analyzed by immunofluorescence using Alexa 568-conjugated antibody (red), lipid droplets were stained with Bodipy 493/503 (green) and nuclei were stained with Hoechst (blue). Results shown are from a representative experiment repeated twice with similar results.

and C/EBP beta, we prepared MEFs, which is another preadipocyte model. We first evaluated the effect of this commercial herbicide on proliferation and found that treatment of cells for 24 h with different dilutions of GF was able to inhibit proliferation of exponentially growing cells in a dose-dependent manner, at similar dilutions of those previously found to be inhibitory in 3T3-L1 fibroblasts [15]. As seen in Figure 3A, GF (dilutions 1:4000 or lower) inhibited the proliferation of exponentially growing cells.

Effect of Glyphosate Formulation on the Differentiation to Adipocytes of MEFs

To confirm the ability of GF to inhibit PPAR gamma induction and adipogenesis, 2 days post-confluent MEFs were induced to differentiate in the presence or absence of GF. Similar concentrations as those found in 3T3-L1 were able to inhibit accumulation of lipids in MEFs as shown in Figure 3B . In addition, the amount of PPAR gamma mRNA was measured by RT-qPCR in MEFs differentiated in the presence of GF during 3 or 7 days, when PPAR gamma is known to be increased, and was found to be impaired with respect to cells treated with DM alone in both cases (Figure 3C).

On the other hand, the amount of the protein C/EBP beta was evaluated in MEFs treated for 24 h with DM in the presence or absence of GF (1:2000 dilution). It was found that 24 h after induction of differentiation C/EBP beta was increased with respect to control cells both in the presence or absence of GF (Figure 3D). These results are in keeping with our findings in 3T3-L1 fibroblasts.

TABLE 1. Effect of Glyphosate Formulation on Lipid Peroxidation

Treatment	*Lipid Peroxidation (Units/μg of Protein)*
Control	1.52 ± 0.13
GF 1:2000	2.63 ± 0.38*
H_2O_2 250 μM	2.05 ± 0.08
H_2O_2 500 μM	3.16 ± 0.06

3T3-L1 cells with 50% confluence were treated for 24 h with medium (C) or with glyphosate formulation final dil 1:2000 (GF 1:2000) or H_2O_2 at the concentrations indicated lipid peroxidation was measured as indicated in section *Materials and Methods*. Results represent mean ± S.E. of two independent experiments. * Significantly different from C, $p < 0.05$ (ANOVA, Tukey's test).

GF-Mediated Increase in Lipid Peroxidation

It has been reported that oxidative stress is involved in the cytotoxic effects of the GF [20, 21]. To evaluate the induction of oxidative stress in 3T3-L1 fibroblasts by GF we analyzed lipid peroxidation after 24 h treatment of exponentially growing cells. Hydrogen peroxide (H_2O_2), which is known to increase ROS and lipid peroxidation in cells [22], was used as a positive control. We found that both GF and H_2O_2 similarly increased lipid peroxidation in these cells (Table 1).

Effect of GF on SOD Activity

In addition, we investigated the effect of GF on SOD activity which is one of the antioxidant enzymes that converts anion superoxide into O_2 and H_2O_2, the latter is then converted to water by glutathione peroxidase. SOD has been reported to be increased by GF in different organisms [20, 23].

We found a significant increase of SOD activity in 3T3-L1 fibroblasts treated for 24 h with GF during exponential growth (Table 2). This increase further suggests that a pro-oxidative state is generated during GF action.

Time- and Dose-Dependent Effect of a Glyphosate-Based Herbicide on Proliferation of Exponentially Growing 3T3-L1 Fibroblasts

Since exposure to glyphosate-based herbicides is usually chronic or repetitive due to agriculture

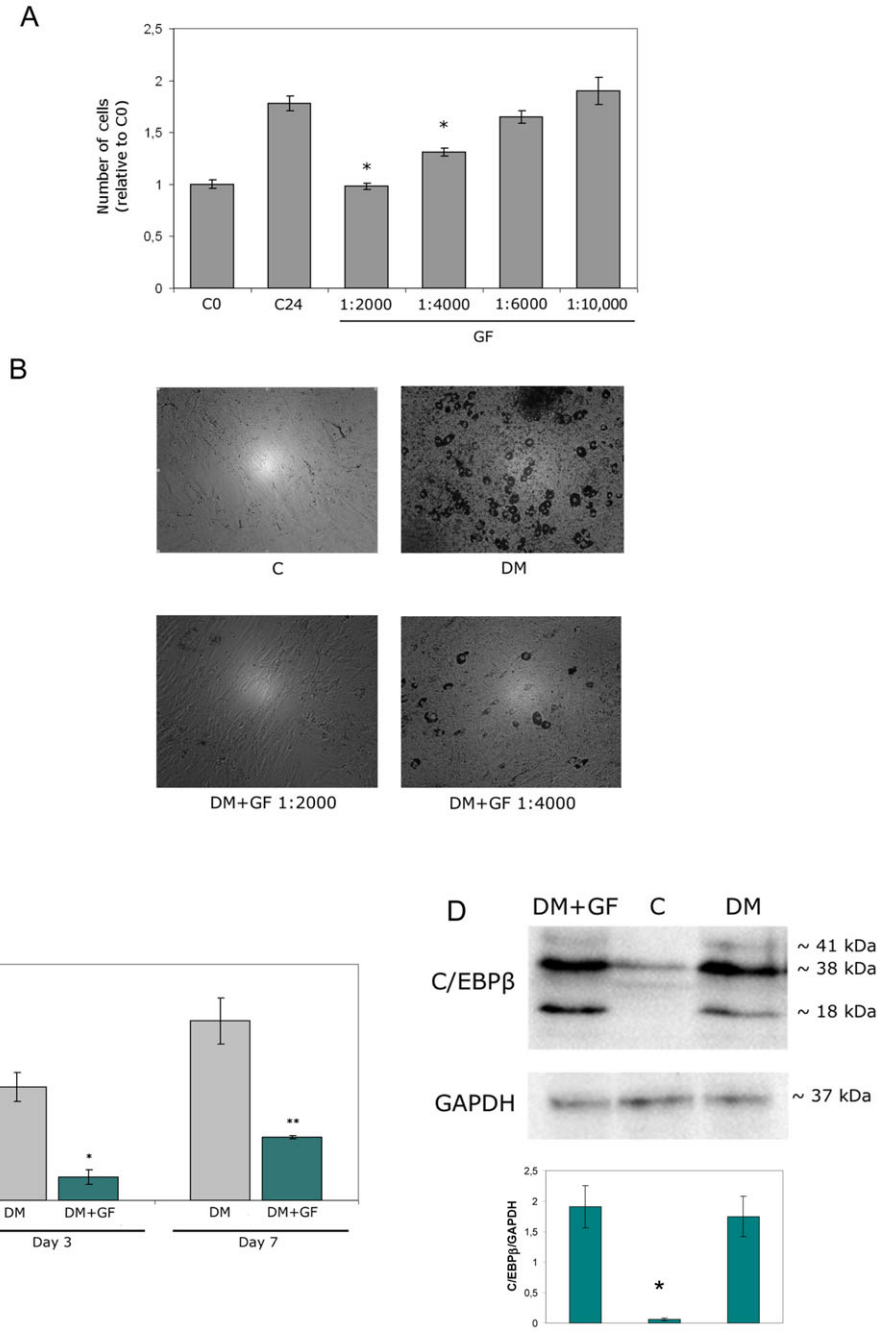

FIGURE 3. Effect of GF on the proliferation and the differentiation of MEFs. (**A**) Cells were cultured in 24-well plates until they reached 30%–40% confluence. At that time, two wells were counted as zero time control (C0). Others were treated for 24 h with medium (C24) or different dilutions of GF as indicated in each case. At the end of these treatments, cells were counted in a Neubauer chamber as indicated in section *Materials and Methods*. Results are expressed relative to C0 which is set to 1 and represent mean ± S.E. of a representative experiment repeated three times with similar results. * Significantly different from C24 $p < 0.05$ (ANOVA, Tukey's test). (**B**) Two days post-confluent cells were treated with: medium alone (C), or with the addition of differentiation mixture (DM), or differentiation mixture plus 1:2000 dilution of GF (DM+GF 1:2000) or 1:4000 dilution (DM+GF 1:4000). GF was maintained in the medium supplemented with insulin that was added 3 days after induction of differentiation. Seven days after induction of differentiation, adipocytes were stained with Oil-Red-O and photographed. Results shown are from a representative experiment repeated three times with similar results. (**C**) The amount of PPAR gamma mRNA was analyzed by RT-qPCR at day 3 or 7 after the induction of differentiation in the presence or absence of 1:2000 GF, as indicated in section *Materials and Methods*. Results shown are from a representative experiment repeated twice with similar results. Data are expressed relative to untreated cells which is set to 1 and represent mean ± S.E. * Significantly different from DM 3 days after induction, $p < 0.05$. ** Significantly different from DM 7 days after induction, $p < 0.05$ (Student's *t*-test). (**D**) 24 h after the induction of differentiation in the presence or absence of GF 1:2000, the amount of C/EBP beta was analyzed by Western blot as indicated in section *Materials and Methods*. Densitometric analysis are shown. Results shown are from a representative experiment repeated three times with similar results. * Significantly different from DM, $p < 0.05$ (ANOVA, Tukey's test).

TABLE 2. Effect of GF on Superoxide Dismutase Activity in 3T3-L1 Fibroblasts

Treatment	*SOD Activity (Units/mg of protein)*
Control	1.81 ± 0.34
GF 1:2000	3.90 ± 0.70*

Cells were cultured in 24-well plates until they reached 50% confluence. At that time, they were treated for 24 h with medium (Control) or with the addition of 1:2000 dilution of GF (GF 1:2000). SOD activity was determined using RANSOD kit, as indicated in section *Materials and Methods*. Results represent mean ± S.E. of three independent experiments. * Significantly different from Control $p < 0.05$ (Student´s *t*-test).

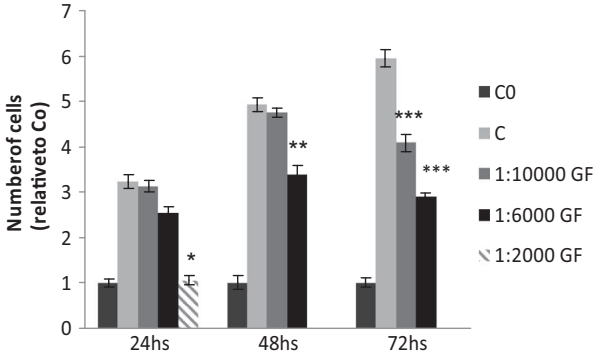

FIGURE 4. Time and dose-dependent effect of GF on 3T3-L1 fibroblasts proliferation. Cells were cultured in 24-well plates until they reached 20% confluence. At that time, two wells were counted as zero time control (C0). Others were treated for 24, 48, or 72 h with medium (C) or different concentrations of GF as indicated in each case. At the end of these treatments, cells were counted in a Neubauer chamber as indicated in methods. Results are expressed relative to C0 which is set to 1 and represent mean ± S.E. of three independent experiments. * Significantly different from C 24 h $p < 0.05$, ** significantly different from C 48 h $p < 0.05$, *** significantly different from C 72 h $p < 0.05$ (ANOVA).

practices, we investigated the effect on proliferation of exponentially growing 3T3-L1 fibroblasts of increasing the time of exposure to concentrations that have no effect after 24 h of treatment. As it is shown in Figure 4, 1:2000 dilution of GF blocked proliferation of exponentially growing 3T3-L1 fibroblasts after 24 h treatment. On the contrary, GF at a dilution 1:6000 did not significantly inhibit proliferation after this time of treatment. However, when treatment with 1:6000 dilution of GF was extended to 48 or 72 h, significant inhibition of proliferation was found in both cases (Figure 4).

In addition, a lower concentration of GF, 1:10,000 dilution, only significantly inhibited proliferation after extending the time of treatment to 72. Considering this fact, it is possible that lower doses than those tested in our assays may become inhibitory at longer time of exposure.

DISCUSSION

In this paper, we found that a commercial glyphosate-based herbicide was able to inhibit proliferation and adipogenesis in both, 3T3-L1 fibroblasts and MEFs. The inhibition of differentiation to adipocytes is due to a decrease in the expression of the master gene in adipogenesis, PPAR gammma but not in C/EBP beta.

Adipogenesis is a coordinated process that starts with the upregulation of C/EBP beta, during the first hours, when mitotic clonal expansion takes place. Later on the cell exits the cell-cycle and adipogenesis starts with the upregulation of PPAR gamma [12, 24]. Accordingly, a dominant-negative PPAR gamma mutant inhibits adipogenesis [24, 25].

Herein, we found that in both, 3T3-L1 and MEFs, GF inhibited the expression of the adipogenic master gene, PPAR gamma, but not C/EBP beta. PPARg inhibition is known to be sufficient to block differentiation. On the other hand, our results show that C/EBP beta was not affected by GF treatment which would suggest that differentiation was triggered, although inhibition by GF of other early adipogenic factors, such as C/EBP delta, could not be discarded.

On the other hand, we found that higher doses of glyphosate alone were needed to inhibit adipogenesis, which is in keeping with previous reports that show the contribution of the adjuvants that are present in commercial formulations [8–11].

In addition, we showed that dilutions of GF up to 1:4000, but not higher, were able to inhibit proliferation in exponentially growing MEFs after 24 h treatment. This is in agreement with our previous findings in 3T3-L1 fibroblasts and with a previous report that showed the ability of Roundup® to block cell cycle progression by inhibiting G2/M transition in sea urchin at a concentration similar to the ones we used in our experiments [7]. These concentrations are subagriculture since suppliers usually suggest dilutions of GF from 1:10 to 1:100. In our experiments, GF was used at subagriculture concentrations in acute treatments, however, lower concentrations might be required for toxic effects in chronic or repetitive exposure to the commercial herbicides as it takes place in the areas planted with glyphosate-resistant crops. Consistently, we found that two concentrations of GF, which were not able to inhibit proliferation of exponentially growing 3T3-L1 fibroblasts after 24 h, became inhibitory after 48 or 72 h treatment. We could not extend longer the time of treatment with the glyphosate-based herbicide because control cells reach confluence and enter into quiescence. Thus, according to our results, potential cytotoxic damage due to chronic exposure to low concentrations of glyphosate-based herbicides could not be discarded. This is in agreement with a recent report where the

administration of very low, environmental concentrations of GF for two years, developed cytotoxic effects in rats [26].

It is well known that oxidative stress is involved in the effect of several pesticides that disrupt cellular physiology [27, 28]. We found that GF was able to induce oxidative stress in exponentially growing 3T3-L1 fibroblasts which was evaluated by the increase in lipid peroxidation and in the activity of SOD, one of the antioxidant enzymes. This is indicative of the ability of GF to alter the redox-state of the cell which is in keeping with previous reports in different organisms [20, 23]. In fact, we analyzed the ability of GF to induce oxidative stress in proliferating 3T3-L1 fibroblasts since the effects of ROS on adipogenesis are controversial. There are reports of ROS inhibiting or enhancing adipogenesis as it has recently been reviewed [29]. ROS are reported to be required for the expression of key adipogenic regulators and the transition of preadipocytes into adipocytes. On the other hand, ROS also act as a harmful agent, disturbing the process of adipocyte differentiation. This might be explained by the fact that tightly regulated, and perhaps specifically localized, levels of ROS are critical for adipogenesis [30]. Our results suggest that GF-mediated oxidative stress may reach those levels that contribute to inhibit adipogenesis although more experiments are needed to prove this point.

In conclusion, a glyphosate-based herbicide was able to inhibit proliferation and differentiation of preadipocytes by downregulating the expression of the key transcription factor of adipogenesis, PPARgamma and to induce oxidative stress which is indicative of its ability to alter cellular physiology.

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