Pioglitazone, a peroxisome proliferator-activated receptor γ agonist, induces cell death and inhibits the proliferation of hypoxic HepG2 cells by promoting excessive production of reactive oxygen species

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Abstract. Hypoxia is a hallmark of solid tumors. Hypoxic cancer cells adjust their metabolic characteristics to regulate the production of cellular reactive oxygen species (ROS) and facilitate ROS-mediated metastasis. Peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear receptor that regulates the transcription of fatty acid metabolism-related genes that have a key role in the survival and proliferation function of hypoxic cancer cells. In the present study, mRNA expression in HepG2 cells under chemically induced hypoxia was assessed. The protein expression levels of hypoxia-inducible factor 1α (HIF-1α) were measured using western blotting. Following treatment with the PPARγ agonist pioglitazone, cell viability was assessed using a Cell Counting Kit-8 assay, whilst cell proliferation and death were determined using 5-ethynyl-2'-deoxyuridine incorporation staining, and calcein-acetoxymethyl ester and propidium iodide staining, respectively. Cellular ROS production was assessed using dihydroethidium staining. Cobalt chloride was used to induce hypoxia in HepG2 cells, which was evaluated using HIF-1α expression. The results revealed that the mRNA expression of PPARγ, CD36, acetyl-co-enzyme A dehydrogenase (ACAD) medium chain (ACADM) and ACAD short-chain (ACADS) was downregulated in hypoxic HepG2 cells. The PPARγ agonist pioglitazone decreased the cell viability of hypoxic HepG2 cells by inhibiting cell proliferation and inducing cell death. Following treatment with the PPARγ agonist pioglitazone, hypoxic HepG2 cells produced excessive ROS. ROS-mediated cell death induced by the PPARγ agonist pioglitazone was rescued with the antioxidant N-acetyl-L-cysteine. The downregulated mRNA expression of PPARγ, CD36, ACADM and ACADS was not reverted by a PPARγ agonist in hypoxic HepG2 cells. By contrast, the PPARγ agonist suppressed the mRNA expression of BCL2, which was upregulated in hypoxic HepG2 cells. In summary, the PPARγ agonist stimulated excessive ROS production to inhibit cell proliferation and increase the death of hypoxic HepG2 cells by decreasing BCL2 mRNA expression, suggesting a negative association between PPARγ and BCL2 in the regulation of ROS production in hypoxic HepG2 cells.

Introduction

Oxidative stress is a condition that involves the excessive production of reactive oxygen species (ROS) to higher levels than antioxidants, and is closely associated with carcinogenesis and cancer progression (1). Excessive ROS production is toxic for cell survival; however, cancer cells exhibit metabolic characteristics enabling them to adjust the antioxidant status and promote ROS-mediated metastasis (2,3). Peroxisome proliferator-activated receptors (PPARs) are a type of nuclear receptor with three members: PPARα, PPARγ and PPARδ. Among them, PPARγ acts as a pivotal regulator of fatty acid degradation. PPARγ activation facilitates fatty acid uptake (4,5), and it has been reported that PPARγ agonists promote the expression of the fatty acid transport protein and acetyl-co-enzyme A (CoA) dehydrogenase (ACAD) medium chain (ACADM) in rat liver (6). PPARγ has also been reported to be downregulated in cells treated with hydrogen peroxide (H_2O_2), which indicates an association between oxidative stress and PPARγ signaling (7). In addition, PPARγ interacts with the Wnt/β-catenin pathway to modulate oxidative stress and promote carcinogenesis (8).

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Hypoxia is a hallmark of solid tumors. Cancer cells reprogram their metabolic characteristics to adapt to hypoxic conditions. Apart from the well-known Warburg effect, fatty acid metabolism reprograming provides energy and macromolecules required for the proliferation, division and survival of cancer cells (9). Fatty acid degradation mainly occurs via β-oxidation, is processed in mitochondria, and is involved in mitochondrial ROS and ATP production and acetyl-CoA recycling (10). β-oxidation is the main form of fatty acid degradation. ACADs consist of ACAD short-chain (ACADS), ACADM, ACAD long-chain and ACAD very-long chain, which degrade short, medium, long and very long-chain fatty acids, respectively (11). Overactive fatty acid β-oxidation is reported to produce ROS and promote metastasis (12). Inhibition of fatty acid oxidation reduces tumor growth and metastasis (13,14); however, ACADM activity suppresses cancer progression (11,15). This conflicting evidence indicates that the role of fatty acid β-oxidation in cancer cells remains elusive.

Oxidative stress and hypoxia are promoters of carcinogenesis, and involve PPARγ. However, the real effect of PPARγ signaling in hypoxic cancer cells remains unclear. Therefore, the present study aimed to assess the effect of the modulation of PPARγ signaling with its agonist, pioglitazone, on hypoxic HepG2 cells.

Materials and methods

Cell culture. HepG2 cells were purchased from the China Center for Type Culture Collection and were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 10 μg/ml streptomycin in an incubator containing 5% CO_2 at 37˚C. The potential presence of mycoplasma in the cell line was detected regularly with a PCR kit (cat. no. C0301S, Beyotime Biotech. Inc., China) followed by the manufacturer's protocol, and no mycoplasma was detected.

Drug administration protocols. The concentration and duration of drug administration depend on the sensitivity and resistance of the cells used. Therefore, the concentration of cobalt chloride ($CoCl_2$) and pioglitazone (Sigma-Aldrich) used in the experiments ranged from 100 to 300 μM and from 10 to 100 μM, respectively (16-21). Based on the literature (16-21), a concentration gradient of $CoCl_2$ and pioglitazone was tested by Cell Counting Kit-8 (CCK-8) assay *in vitro* (data not shown). Cells were then treated with $CoCl_2$ (200 μM) and pioglitazone (40 μM) for 24, 48 or 72 h.

CCK-8 assay. HepG2 cells were seeded into a 96-well plate and cultured overnight. Before treatment, cells were replenished with medium and treated with different concentrations of $CoCl_2$ ranging from 100 to 300 μM. When the treatment ended at 24 h or 48 h, 10 μl CCK-8 reagent (Vazyme Biotech Co., Ltd.) was added to each well, and the plate was incubated at 37˚C for 1 h. Subsequently, the absorbance at 450 nm was measured using a microplate reader (SpectraMax i3x; Molecular Devices, LLC). The optical density of each well was normalized to the control group and calculated.

Reverse transcription (RT)-quantitative (q)PCR. Gene expression levels were quantified by RT-qPCR. Total RNA was isolated from cells using TRI Reagent® (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. The concentration of RNA was determined using NanoDrop™ 2000 (Thermo Fisher Scientific, Inc.). cDNA was synthesized from 0.5-2.5 μg RNA by using the HiScript® II 1st Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd.) at 42˚C for 5 min, 37˚C for 15 min and 85˚C for 5 sec, performed on a T100 thermocycler (Bio-Rad Laboratories, Inc.). Gene expression was determined by qPCR using specific primers and SYBR Green (Vazyme Biotech Co., Ltd.) in a QuantStudio™ 3 system (Thermo Fisher Scientific, Inc.) with the following thermocycling conditions: Denaturing at 95˚C for 10 min, followed by 40 cycles of denaturing at 95˚C for 15 sec and annealing and extension at 60˚C for 1 min. Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (22). The primers used for qPCR are listed in Table I. Gene expression was measured in duplicate and was normalized using ribosomal protein S18 as the housekeeping gene.

Western blotting. Protein samples were prepared in lysis buffer (25 mmol/l HEPES, 150 mmol/l potassium acetate, 2 mmol/l EDTA pH 8.0, 0.1% NP-40, 10 mmol/l sodium fluoride, 50 mmol/l PMSF, 1 μg/μl aprotinin, 1 μg/μl pepstatin, 1 μg/μl leupeptin and 1 mmol/l dithiothreitol). The protein determination method was the BCA protein assay (Beyotime Institute of Biotechnology), which was performed according to the manufacturer's protocol using bovine serum albumin (BSA; Servicebio, Ltd.) to prepare a standard curve. SDS-PAGE was performed using 10-20 μg protein/lane and 4-15% gels (Beyotime Institute of Biotechnology), followed by transblotting to a 0.2 μm nitrocellulose membrane (Amersham; Cytiva). The membranes were blocked with 5% skimmed milk (in buffer containing 10 mM Tris pH 8.0, 150 mM NaCl and 0.05% Tween 20) for 1 h at room temperature. The membranes were incubated overnight with primary antibody at 4˚C and then incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Protein band intensities were determined and detected with BeyoECL Star (Beyotime Institute of Biotechnology) using the Amersham™ Imager 680 system (Amersham; Cytiva). The primary antibodies included anti-hypoxia-inducible factor (HIF)-1α (rabbit monoclonal antibody (mAb); cat. no. 36169S; Cell Signaling Technology, Inc.) and β-actin (rabbit mAb; cat. no. 4970S; Cell Signaling Technology, Inc.), diluted 1:1,000 in 1% BSA. The secondary antibody was HRP-conjugated goat anti-rabbit immunoglobulin G (cat. no. 5127S; Cell Signaling Technology, Inc.)) diluted 1:1,000.

Fluorescence microscopy

Live and dead cell staining. Live and dead cell staining was performed using the Live & Dead Kit (BioScience) following the manufacturer's instructions. HepG2 cells treated in the presence or without 200 μM CoCl2 (Co), 40 μM pioglitazone (P) and both (Co + P) for 72 h, or HepG2 cells treated with or without 200 μM CoCl2 (Co), 40 μM pioglitazone (P) and both (Co + P) for 48 h. At the end of the treatment, cells were replenished with serum-free DMEM containing

Table I. Primers for reverse transcription-quantitative PCR.

Gene	Forward primer, 5'→3'	Reverse primer (5'-3')
RPS18	TGCGAGTACTCAACACCAACA	CTTCGGCCCACACCCTTAAT
PPARγ	AGAGCCTTCCAACTCCCTCA	TCTCCGGAAGAAACCCTTGC
CD36	TGTGCAAAATCCACAGGAAGTG	GGCTAGAAAACGAACTCTGTACG
ACADM	GGGTTCGGGCGATGCTG	CTGCTGTTCGGTGAACTCAAA
ACADS	TGAATGGAACCAAAGCCTGGA	AGGCACTGATGCCCTTGTTTT
HMOX1	ACCTTCCCCAACATTGCCAG	CAACTCCTCAAAGAGCTGGATG
BCL2	AGATTGATGGGATCGTTGCCT	AGTCTACTTCCTCTGTGATGTTGT

RPS18, ribosomal protein S18; PPARγ, peroxisome proliferator-activated receptor γ; ACAD, acetyl-co-enzyme A dehydrogenase; ACADM, ACAD medium-chain; ACADS, ACAD short-chain; HMOX1, heme oxygenase 1; BCL2, B cell lymphoma-2.

2 μM calcein-acetoxymethyl ester (calcein-AM) and 4.5 μM propidium iodide (PI), and then incubated for another 30 min at 37˚C. The plate was observed under a fluorescence microscope (Leica DFC450; Leica Microsystems, Inc.). The integrated density of calcein-AM and PI was analyzed using ImageJ software (National Institutes of Health) version 1.54.

5-Ethynyl-2'-deoxyuridine (EdU) staining. EdU staining was performed using the BeyoClick EdU-488 kit (Beyotime Institute of Biotechnology) following the manufacturer's instructions. Cells were seeded into coverslips, and then cultured and treated as aforementioned. A total of 10 μM EdU solution was added to the culture medium 6 h before the end of treatment. After incorporation of EdU, coverslips were washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Coverslips were then washed again with PBS and incubated with 0.3% Triton (Beyotime Institute of Biotechnology) for 10 min at room temperature. After washing with PBS, coverslips were incubated with BeyoClick-reactive solution containing labeled azide for 30 min at room temperature. Coverslips were washed with PBS, sealed with mounting medium containing DAPI and then observed under a fluorescence microscope (Olympus ix73; Olympus Corporation). Cell count was carried out with ImageJ software (National Institutes of Health) version 1.54 using manual and automatic methods.

ROS staining. ROS staining was performed using a DHE (Dihydroethidium) assay (Abcam.) following the manufacturer's instructions. Cells were cultured and treated as aforementioned. At the end of treatment, cells were replenished with serum-free DMEM containing 5 μM DHE. Cells were cultured for another 30 min at 37˚C and then observed under a fluorescence microscope (Leica DFC450; Leica Microsystems, Inc.). Integrated fluorescence density was analyzed using ImageJ.

Statistical analysis. Data are presented as the mean ± standard deviation from ≥3 independent experiments. Data analysis was performed using GraphPad Prism (version 7; GraphPad; Dotmatics). Multiple group comparisons were first performed with analysis of variance (one-way ANOVA), followed by Bonferroni post hoc test if the results of ANOVA achieved statistical significance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PPARγ-associated fatty acid metabolism genes are downregulated in hypoxic HepG2 cells. $CoCl_2$ has been widely used to induce the hypoxia of cultured cells *in vitro* (23). In the present study, $CoCl_2$ was used to imitate the hypoxic conditions in which HepG2 cells could survive. The maximum dose (400 μM)of $CoCl_2$ that maintained >80% cell viability was determined using a CCK-8 assay (Fig. 1A). The expression of the HIF-1α protein was notably increased in a dose-dependent manner in $CoCl_2$-treated HepG2 cells, which demonstrated that HepG2 cells were under hypoxic conditions (Fig. 1B). The expression of the antioxidant gene heme oxygenase 1 (HMOX1) was significantly increased ~10-fold higher in hypoxic HepG2 cells treated with 400 μM $CoCl_2$ than control HepG2 cells (Fig. 1C). By contrast, the mRNA expression of PPARγ was significantly downregulated about 50% in HepG2 cells treated with 400 μM $CoCl_2$ (Fig. 1C). PPARG is the gene encoding PPARγ (24). In-line with the downregulation of PPARγ, several key regulator genes associated with fatty acid metabolism, including CD36, ACADM and ACADS (11), were significantly downregulated in HepG2 cells treated with 400 μM $CoCl_2$ than control HepG2 cells (Fig. 1C). These results indicated that downregulation of PPARγ was accompanied by an increased severity of hypoxia in HepG2 cells.

PPARγ agonist pioglitazone decreases cell proliferation and induces cell death in hypoxic HepG2 cells. To determine whether the activation of PPARγ promoted the death of hypoxic HepG2 cells, pioglitazone, an established PPARγ agonist (25), was added to activate PPARγ signaling in hypoxic HepG2 cells. Pioglitazone (20-60 μM) in combination with 200 μM $CoCl_2$ significantly reduced the viability of HepG2 cells treated with 200 μM $CoCl_2$ alone (Fig. 2A). Following treatment with pioglitazone, the proliferation of hypoxic HepG2 cells was further inhibited, and markedly few EdU^+ cells were observed following treatment with a combination of $CoCl_2$ and pioglitazone compared with cells treated with $CoCl_2$ alone (Fig. 2B). The EdU^+ cell ratio of hypoxic HepG2 cells was calculated, and the results revealed that pioglitazone significantly inhibited the proliferation of hypoxic HepG2 cells treated with $CoCl_2$ (Fig. 2C). In the combination group of hypoxia and pioglitazone, the number of calcein-AM^+ cells and

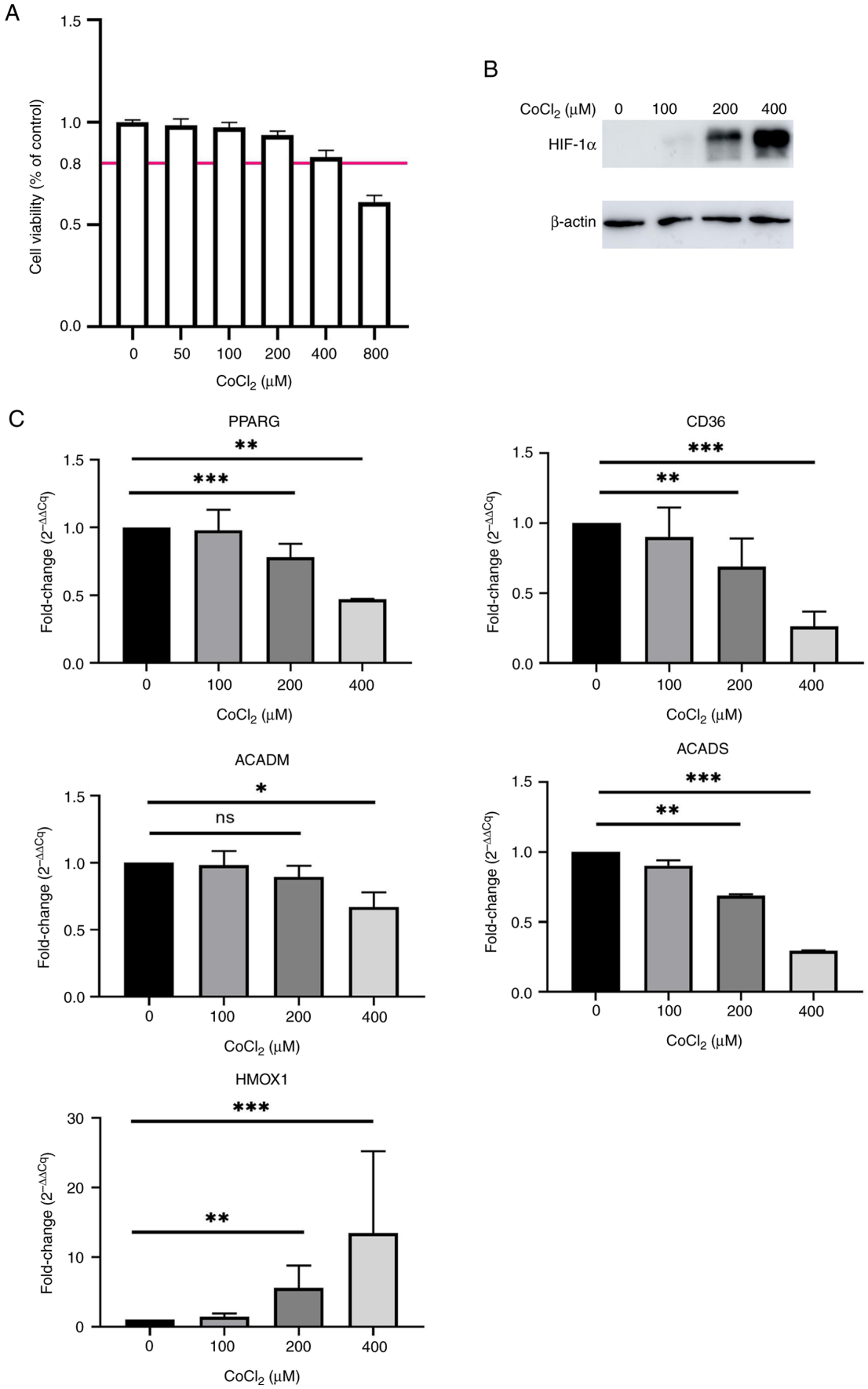

Figure 1. mRNA expression of PPARγ is downregulated in $CoCl_2$-treated HepG2 cells. (A) Viability of HepG2 cells treated with a gradient dose of $CoCl_2$ for 24 h. (B) Protein expression of HIF-1α in HepG2 cells treated with different doses of $CoCl_2$ for 24 h. β-actin was used as a loading control. (C) mRNA expression of PPARγ, CD36, ACADM, ACADS and HMOX1 in HepG2 cells treated with different doses of $CoCl_2$ for 24 h (n=3-4). *P<0.05; **P<0.01; ***P<0.001. PPARγ, peroxisome proliferator-activated receptor γ; HIF-1α, hypoxia-inducible factor-1α; ACAD, acetyl-co-enzyme A dehydrogenase; ACADM, ACAD medium-chain; ACADS, ACAD short-chain; HMOX1, heme oxygenase 1; ns, not significant; $CoCl_2$, cobalt chloride; Cq, quantification cycle.

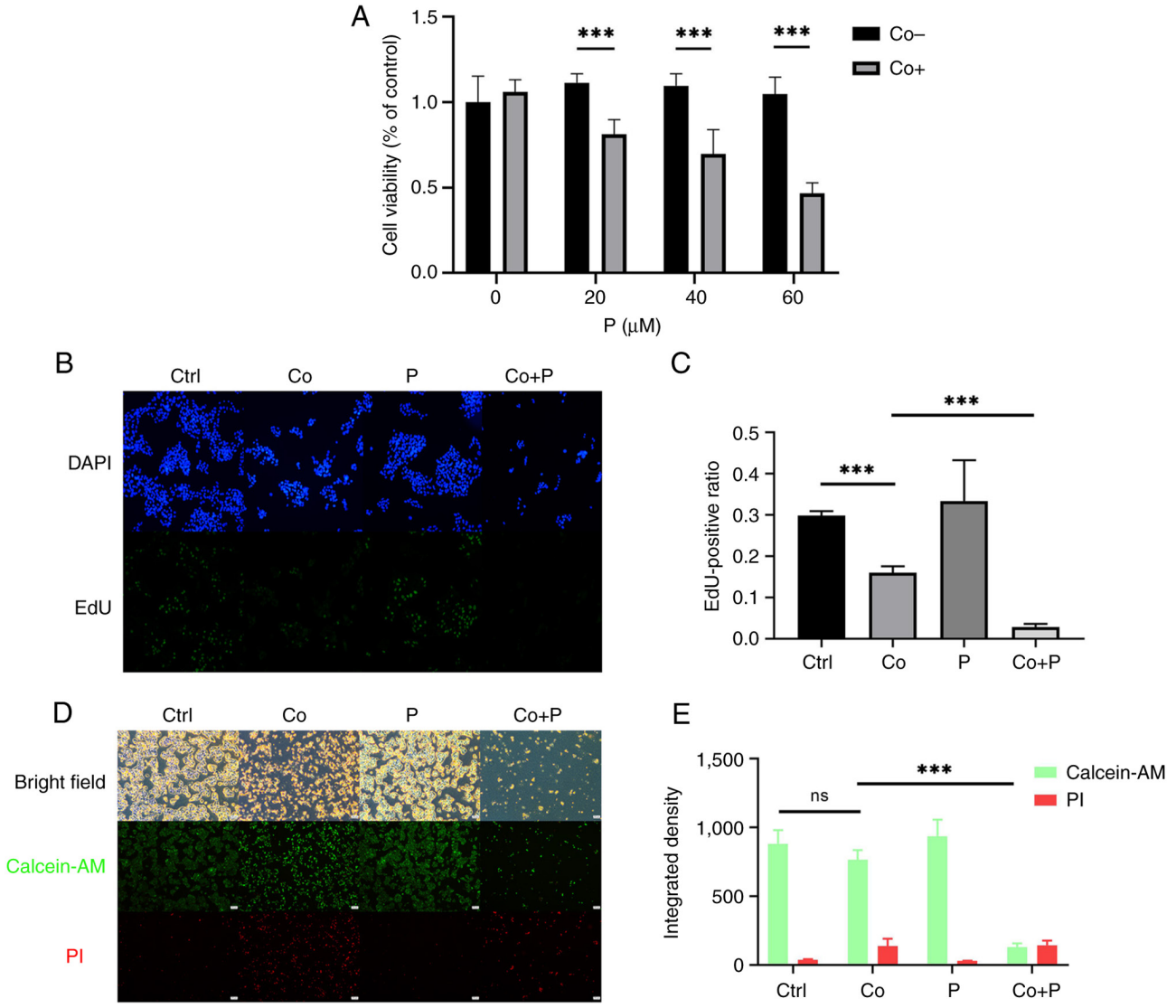

Figure 2. A PPARγ agonist inhibits the proliferation and promotes the death of hypoxic HepG2 cells. (A) HepG2 cells were treated with 20, 40 and 60 μM P with or without 200 μM Co for 48 h. P was added 24 h before Co. Cell viability was measured using a Cell Counting Kit-8 assay. (B) EdU staining of HepG2 cells treated with or without 200 μM Co, 40 μM P, and both Co + P for 48 h (magnification, x40). P was added 24 h before Co. (C) EdU$^+$ cells were counted using ImageJ software version 1.54, and the positive ratio was compared between different groups. (D) Calcein-AM and PI double staining of HepG2 cells treated with or without 200 μM Co, 40 μM P and both Co + P for 72 h (scale bar, 500 μm). P was added 24 h before Co. (E) Integrated density of calcein-AM and PI was calculated using ImageJ (n=3-4). ***P<0.001. PPARγ, peroxisome proliferator-activated receptor γ; P, pioglitazone; Co, cobalt chloride; EdU, 5-ethynyl-2'-deoxyuridine; Ctrl, control; calcein-AM, calcein-acetoxymethyl ester; PI, propidium iodide; ns, not significant; BF, bright field.

their fluorescence intensity were notably reduced compared with those in HepG2 cells treated with hypoxia only (Fig. 2D). The integrated density of calcein-AM and PI was calculated in repeated staining experiments. The cell confluence was low in the CO+P. PI staining cannot stain the detached dead cells. With optimization of treatment time, the CO + P showed the most dead cells, and the results suggested that pioglitazone in combination with $CoCl_2$ promoted the death of HepG2 cells treated with $CoCl_2$ alone (Fig. 2E). These results demonstrated that the activation of PPARγ abolished the survival and proliferation abilities of hypoxic HepG2 cells.

PPARγ agonist pioglitazone induces the death of hypoxic HepG2 cells via aggravation of oxidative stress. In the present study, pioglitazone was demonstrated to induce the death of hypoxic HepG2 cells treated with $CoCl_2$. Given the PPARγ agonist pioglitazone increased intracellular ROS production and then exacerbated the oxidative stress of hypoxic HepG2 cells, it was hypothesized that supplementation of an antioxidant could rescue hypoxic HepG2 cells. To assess this hypothesis, N-acetyl-L cysteine (NAC) was added to scavenge intracellular ROS in hypoxic HepG2 cells treated with pioglitazone. Following addition of NAC, the number of calcein-AM positive cells was markedly increased, whilst the number of PI positive cells was notably decreased compared to cells treated with Co + P (Fig. 3A). Furthermore, following NAC treatment, the integrated fluorescence intensity of PI of cells was significantly diminished compared with cells treated with Co + P (Fig. 3C). DHE staining was then performed to directly assess intracellular ROS production in HepG2 cells. The staining results demonstrated that increased red fluorescence in the Co + P group

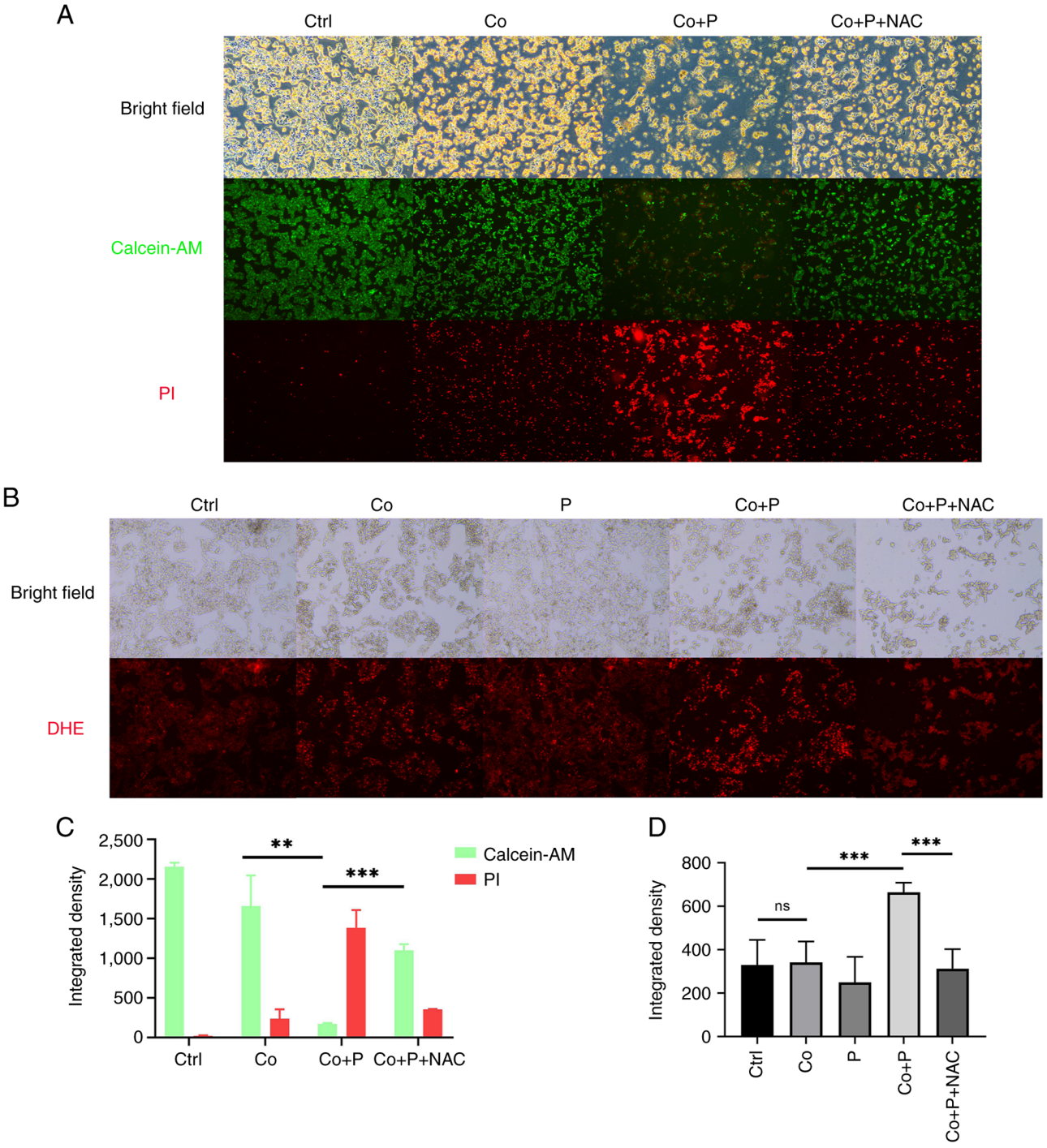

Figure 3. A PPARγ agonist increases the levels of intracellular reactive oxygen species to induce cell death. (A) Calcein-AM and PI double staining of HepG2 cells treated with or without 200 μM Co, 40 μM P and both Co + P for 48 h (magnification, x50). (B) DHE staining of HepG2 cells treated with or without 200 μM Co, 40 μM P and both Co + P for 48 h (magnification, x50). Integrated density of fluorescence was measured and calculated in samples of panels (C) A and (D) B. (n=3-4). **P<0.01; ***P<0.001. PPARγ, peroxisome proliferator-activated receptor γ; calcein-AM, calcein-acetoxymethyl ester; PI, propidium iodide; P, pioglitazone; Co, cobalt chloride; NAC, N-acetyl-L cysteine; DHE, dihydroethidium; Ctrl, control; ns, not significant; BF, bright field.

vs. Co group, indicated the PPARγ agonist notably increased intracellular ROS in hypoxic HepG2 cells, which could be scavenged by NAC (Fig. 3B). The comparison of quantified integrated fluorescence density revealed that the PPARγ agonist significantly increased integrated fluorescence in the Co + P group vs. Co group, whilst NAC significantly reduced integrated fluorescence in the Co + P + Nac group vs. Co + P group (Fig. 3D). The results demonstrated that the PPARγ agonist pioglitazone induced cell death through the production of excessive ROS and the induction of oxidative stress in hypoxic HepG2 cells.

PPARγ agonist-mediated induction of ROS production may not occur through fatty acid oxidation but through impairment of B-cell lymphoma-2 (BCL2) expression. Fatty acid oxidation has been reported to be a source of ROS in cancer cells (17). Therefore, whether PPARγ increased intracellular ROS production in hypoxic HepG2 cells was assessed by enhancing

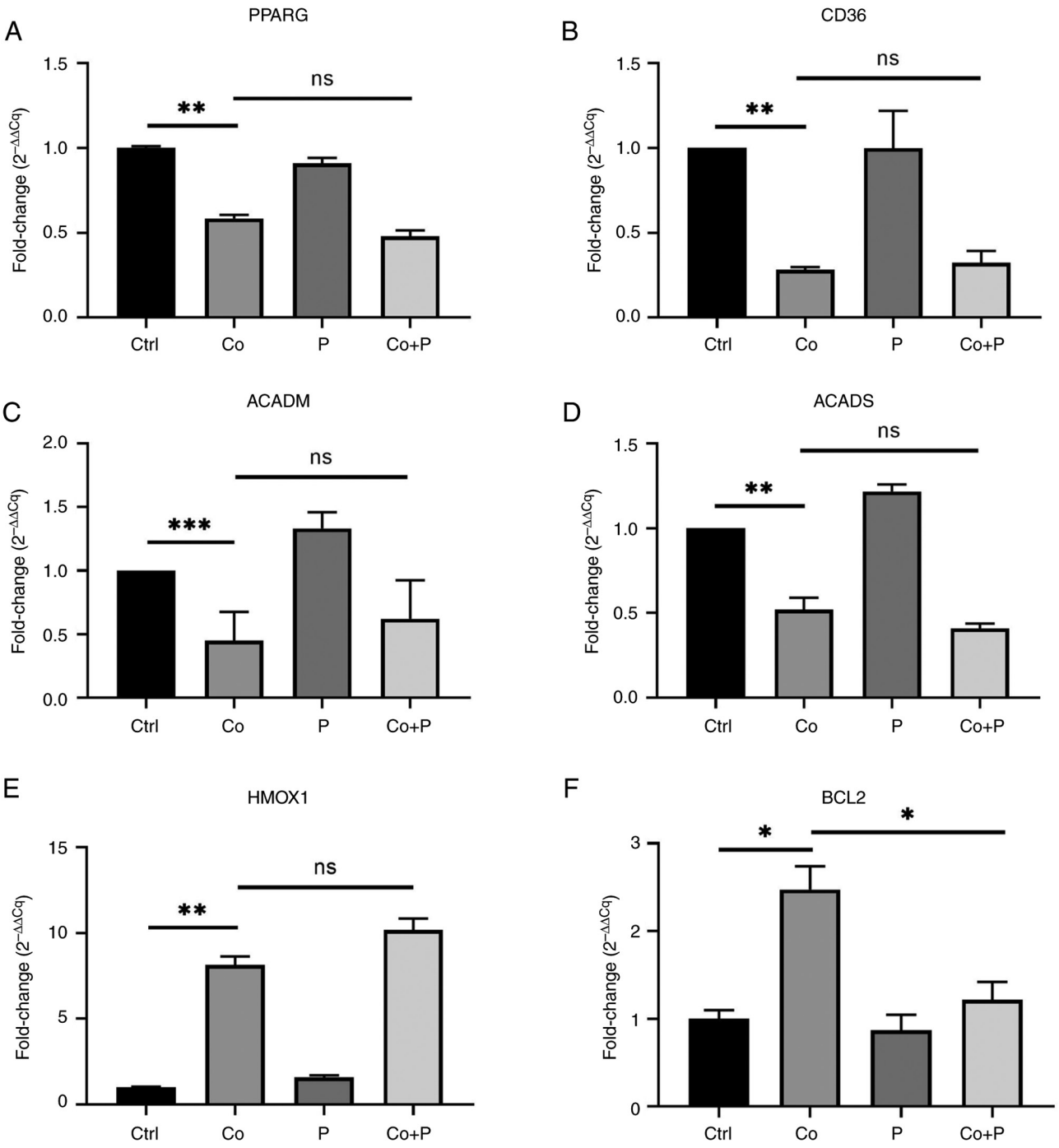

Figure 4. A PPARγ agonist downregulates anti-apoptotic gene expression but does not revert downregulated expression of fatty acid metabolism-related genes. HepG2 cells were treated with or without 200 μM Co, 40 μM P or both Co + P for 48 h. The mRNA expression of (A) PPARγ, (B) CD36, (C) ACADM, (D) ACADS, (E) HMOX1 and (F) BCL2 was measured and analyzed (n=3-4). *P<0.05; **P<0.01; ***P<0.001. PPARγ, peroxisome proliferator-activated receptor γ; Co, cobalt chloride; P, pioglitazone; ACAD, acetyl-co-enzyme A dehydrogenase; ACADM, ACAD medium-chain; ACADS, ACAD short-chain; HMOX1, heme oxygenase 1; BCL2, B-cell lymphoma-2; Ctrl, control; ns, not significant; Cq, quantification cycle.

fatty acid oxidation. The mRNA expression of PPARG, CD36, ACADM and ACADS was measured. The expression of genes including PPARG, CD36, ACADM and ACADS significantly reduced about 50% in HepG2 cells treated with $CoCl_2$. However, the downregulated expression of these genes was not reverted by the PPARγ agonist (Fig. 4A-D). In addition, the expression of the antioxidant gene HMOX1 was also measured to evaluate the antioxidant reactivity of hypoxic HepG2 cells treated with the PPARγ agonist. However, the results revealed that the PPARγ agonist did not significantly stimulate the expression of HMOX1 in Co + P group compare to Co group (Fig. 4E).

BCL2 has been reported to be a key regulator of mitochondrial ROS production, and is able to confer anti-apoptotic effects to cancer cells with oxidative stress (18). Therefore, whether the aforementioned PPARγ agonist regulated intracellular ROS production by modulating BCL2 expression was assessed. The mRNA expression of BCL2 was measured, and

the data indicated that hypoxic HepG2 cells had a significantly higher expression of BCL2 than control cells, and the PPARγ agonist significantly decreased the expression of BCL2 in Co + P group cells compared to Co group cells (Fig. 4F). These results indicated that the PPARγ agonist pioglitazone increased intracellular ROS production in hypoxic HepG2 cells, not via upregulation of the expression of fatty acid degrading genes, but via downregulation of the expression of the anti-apoptotic gene BCL2.

Discussion

The generation of a hypoxic environment and the activation of HIF-1 are common features of advanced cancer (26). The response to hypoxia is mainly attributed to HIFs. $CoCl_2$-induced chemical hypoxia is one of the most commonly used models of cell hypoxia *in vitro*, and the use of $CoCl_2$ *in vitro* has been reported to increase HIF-1α/2α in a dose-and time-dependent manner (23). $CoCl_2$ strongly stabilizes HIFs, thereby mimicking hypoxia and inducing the upregulation of a range of hypoxic adaptive responses, many of which have potential carcinogenic effects (27). As alteration of the cellular adaptation to hypoxia is also fundamental in cancer treatment (26), it was hypothesized that it would be meaningful to assess the mechanism and to identify an approach to abolish the resistance of cells to $CoCl_2$. Nevertheless, the hypoxic response can also be detrimental for tumorigenesis. Cobalt is cytotoxic and induces apoptosis and necrosis at high concentrations (27). Horev-Azaria *et al* (28) reported that $CoCl_2$ decreased the viability of NCI-H441 cells by 30-40% at 0.4 mM after 48 and 72 h, and of HepG2 cells by 70-100% at 0.4 mM after 48 and 72 h via MTT assay.

The experiments carried out in the present study revealed that the downregulation of the expression of PPARG and fatty acid oxidation genes was accompanied by increased expression of HIF-1α in hypoxic HepG2 cells. The PPARγ agonist pioglitazone specifically stimulated excessive ROS production in hypoxic HepG2 cells to induce cell death and inhibit cell proliferation. Notably, the effect of this PPARγ agonist did not depend on fatty acid oxidation, but on the downregulation of BCL2 expression in hypoxic HepG2 cells. Thus, the results of the current study reveal a negative association between PPARγ and BCL2 in regulating ROS production in hypoxic HepG2 cells.

Under hypoxic conditions, cancer cells dynamically modulate the intake and degradation of fatty acids to balance ROS production (11,14,29). Fatty acid degradation mainly occurs via β-oxidation and is catalyzed by ACADs. Fatty acid oxidation can be regulated in numerous tissues by activating the PPAR signaling pathway. PPARs are nuclear receptors that regulate lipid metabolism by promoting gene transcription (30,31). It has been reported that PPARγ serves an important role in tumor cell proliferation and death, as well as in angiogenesis, invasion and metastasis (32). Once activated by a ligand, PPARγ binds to DNA-specific PPAR response elements and modulates the transcription of its target genes, such as CD36, ACADM and ACADS, which may be important regulatory targets of fatty acid metabolism in tumor cells. CD36 stimulates tumor development and metastasis by allowing cells to absorb lipids from the extracellular environment, and promotes fatty acid synthetase oxidation to produce ATP (33,34). CD36 is expressed at a high level in glioblastoma, and the reduction in CD36 leads to a loss of self-renewal and tumor initiation ability (35). ACADM reflects fatty acid metabolism and gemcitabine sensitivity in pancreatic cancer, potentially providing a reliable way to measure the efficacy of chemotherapy (36). ACADS is a potential methylation biomarker associated with the proliferation and metastasis of hepatocellular carcinoma (37). PPARγ is the key regulator of ACADM transcription, which first catalyzes the reaction of the β-oxidation cycle for 4-10-carbon fatty acids (6). PPARγ signaling is dysregulated in cancer cells under hypoxia or oxidative stress (7,38). In the present study, it was hypothesized that the association between PPARγ and fatty acid oxidation may be essential for ROS regulation in hypoxic cancer cells. PPARγ and its downstream genes, including CD36, ACADM and ACADS, were all downregulated in hypoxic HepG2 cells. HMOX1 is a key enzymes to antagonize harmful ROS of cytoplasm (39). The marked increase in HMOX1 gene expression in hypoxic HepG2 cells observed in the current study suggests a cellular adjustment of the redox status to regulate ROS production.

Pioglitazone is an established PPARγ agonist, and is approved for the clinical treatment of diabetes. Despite its effect on glucose metabolism, pioglitazone has been reported to enhance fatty acid β-oxidation (25). Given that impaired PPARγ activity confers cancer cells tolerance to hypoxia, activation of PPARγ signaling should disrupt the intracellular metabolic balance of hypoxic HepG2 cells. The results of the present study revealed that pioglitazone decreased the viability of hypoxic HepG2 cells by inhibiting cell proliferation and inducing cell death. However, the effect of PPARγ in hypoxia is controversial. It has been reported that the PPAR agonist pioglitazone protects against hypoxia-induced fetal growth inhibition (40). Similarly, another PPARγ agonist has been reported to have a protective effect against hypoxia in cardiac myocytes (41). However, inhibition of PPARγ and HIF-1α potentiated the sensitivity of HepG2 cells to a tyrosine kinase inhibitor (42). It could be assumed that the conflicting PPARγ effect in hypoxia may be due to differences in metabolic characteristics between cancer and benign cells. In the present study, it was demonstrated that the PPARγ agonist pioglitazone abolished the hypoxia tolerance of HepG2 cells.

Oxidative stress is a status of excessive ROS compared with antioxidants. Cancer cells have aberrant redox homeostasis to tolerate high ROS levels, and adjust their antioxidant status to facilitate ROS-driven proliferation and to avoid ROS-induced senescence, apoptosis or ferroptosis (1). Glutathione (GSH) is a fast reactive endogenous antioxidant that scavenges intracellular ROS (43). Cysteine is the necessary source of GSH biosynthesis, and NAC is widely used as the source of GSH production (44). It can be hypothesized that the PPARγ agonist pioglitazone induces the death of hypoxic HepG2 cells by increasing the production of excessive ROS, and the effect of pioglitazone may be neutralized by NAC-stimulated GSH production. The results of the present study demonstrated that the above PPARγ agonist specifically increased intracellular ROS production in hypoxic HepG2 cells. By contrast, NAC protected hypoxic HepG2 cells against this PPARγ agonist by scavenging intracellular ROS and

reducing PPARγ agonist-mediated cell death. An association between the PPARγ agonist pioglitazone and excessive ROS production was also demonstrated in hypoxic HepG2 cells. Similarly, this PPARγ agonist has been reported to increase intracellular ROS production in lung cancer cells exposed to γ-radiation (45). Nevertheless, pioglitazone has been reported to inhibit the ROS production of cardiac fibroblasts treated under anoxia-reoxygenation conditions (46). Therefore, it can be hypothesized that the diverse effects of this PPARγ agonist result from the dysregulated redox status of hypoxic cancer cells.

Mitochondria are the main producers of cellular ROS, including superoxide and/or H_2O_2, via aerobic metabolism (47). ROS production is tightly controlled, and HIFs alter the structure and activity of the electron transport chain to regulate ROS production under hypoxia (48). Stimulating fatty acid oxidation has been reported to disturb the redox balance of hypoxic cancer cells. Furthermore, increased expression of ACADM has been reported to decrease cell proliferation and invasion in solid tumors, whilst impaired ACADM activity promotes cancer progression (11,15). In the present study, it was demonstrated that the mRNA expression of fatty acid-degrading genes, including PPARG, ACADM and ACADS, was downregulated, and the PPARγ agonist pioglitazone promoted excessive ROS production in hypoxic HepG2 cells. However, the results of the present study do not suggest that the PPARγ agonist upregulated fatty acid oxidation in hypoxic HepG2 cells. PPARγ expression has been reported to be dysregulated in esophageal cancer, and a PPARγ agonist inhibited cancer cell proliferation *in vitro* and cancer progression *in vivo* via the Akt-$P21^{CIP1}$ signaling pathway (49). Moreover, the PPARγ agonist lobeglitazone has been reported to inhibit thyroid cancer cell metastasis by suppressing the MAPK signaling pathway (50). The evidence implies that the effect of PPARγ agonists is not confined to modulating fatty acid metabolism. Moreover, the PPARα activator fenofibrate has been reported to induce apoptosis in human hepatocellular carcinoma cells by increasing ROS production (51). In view of PPARα-dependent apoptosis, PPARα can be considered an E3 ubiquitin ligase able of inducing the ubiquitination and degradation of BCL2, thus leading to apoptosis (52). BCL2 is a mitochondrial membrane protein that regulates ROS production and serves as an anti-apoptotic effector in the endogenous apoptosis pathway (53). Therefore, downregulation of BCL2 by PPARγ agonists stimulates excessive ROS production in hypoxic HepG2 cells. The results of the present study indicate a negative association between BCL2 expression and PPARγ activity in hypoxic HepG2 cells. This mechanism appears to be essential for regulating ROS production in hypoxic HepG2 cells.

In conclusion, PPARγ downregulation appears to be required for redox homeostasis in hypoxic HepG2 cells. In the present study, the PPARγ agonist pioglitazone stimulated excessive ROS production in hypoxic HepG2 cells to induce cell death and inhibit cell proliferation via downregulation of BCL2 expression. Notably, differences between lack of oxygen-induced hypoxia and $CoCl_2$-induced chemical hypoxia may lead to proapoptotic responses, and the genes induced by the two modes of hypoxia may not overlap. However, other anti-apoptotic genes potentially modulated by pioglitazone were not included in the present study, and therefore further experiments are needed to fully assess the role of pioglitazone in $CoCl_2$-induced hypoxic HepG2 cells.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

GH conceived the study, performed the experiments, and analyzed and interpreted the data. MZ conceived the study and analyzed and interpreted the data. MW and WX performed the experiments. XD and XH interpreted the data and revised the manuscript. XD and MZ confirm the authenticity of all the raw data. JR and MZ funded the study, and interpreted the data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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