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A modified fixed staining method for the simultaneous measurement of reactive oxygen species and oxidative responses

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ABSTRACT

The generation of reactive oxygen species (ROS) in a live-cell system is routinely measured using the oxidation-sensitive fluorescent probe dichlorofluorescein (DCF). However, it is difficult to simultaneously monitor cellular oxidative responses and ROS generation in cells, and analyses of cellular oxidative responses are typically performed after ROS generation has been evaluated. In this study, we developed a modified fixed staining method that allows the simultaneous analysis of ROS generation and oxidative responses using standard immunostaining techniques. A microplate reader-based assay showed that of the fixatives tested, only methanol did not alter the hydrogen peroxide (H_2O_2)-mediated oxidation of the responsive dye 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), a chloromethyl derivative of H_2DCFDA, or the fluorescence of oxidized DCF *in vitro*. Further *in vivo* assays using flow cytometry showed that both methanol and acetic acid maintained the fluorescence of oxidized DCF in H_2O_2-, antimycin A-, and serum starvation-treated human lung adenocarcinoma A549 cells and human microvascular endothelial HMEC-1 cells. Following acetic acid-based fixation, the ROS generation in starved HMEC-1 cells could be evaluated by flow cytometric analysis while simultaneously monitoring the phosphorylation status of p38 mitogen-activated protein kinase. Immunostaining also revealed the synchronization of ROS generation and the H_2O_2-induced phosphorylation of Src homology-2 domain-containing phosphatase 2. This study describes a modified method that may be used in future biomedical investigations to simultaneously measure intracellular ROS production and cellular oxidative responses.

1. Introduction

Reactive oxygen species (ROS) are chemically unstable molecules that are derived from oxygen and are mainly produced during oxidative phosphorylation via the mitochondrial respiratory chain complexes I and III [1]. ROS levels can be elevated by physiological and pathological stimuli, which determine the degree of cellular oxidative stress. During normal cell respiration, approximately 6% of the oxygen reduced in the mitochondria is converted to superoxide radicals [2]. Superoxide radicals are converted to hydrogen peroxide (H_2O_2) by superoxide dismutase [3] and are subsequently converted to other types of ROS, including hydroperoxyl radicals, hydroxyl radicals, peroxyl radicals, alkoxyl radicals, and reactive nitrogen species [4]. Due to the varying oxidative reactivities and short half-lives of ROS, studies of ROS generation can be very challenging. Among the major ROS, H_2O_2 exhibits the lowest reactivity and highest stability and is present at the highest intracellular concentrations [5].

Oxidative stress-induced cellular activation is generally caused by the ROS-mediated oxidation of DNA [6], polyunsaturated fatty acids in lipids [7], and amino acids in proteins [8]. In addition to the direct effects of oxidation, cellular oxidative responses, such as the activation of mitogen-activated protein kinase (MAPK) cascades, including the extracellular signal-related kinases (ERK) [9], the c-Jun N-terminal kinases (JNK) [10], and p38 MAPK [11], are required for cellular responses through their downstream signaling pathways [12,13]. Recent studies have shown that ROS promote activation of the Src homology-2 domain-containing phosphatase (SHP) 2 [14,15], which is essential for Ras/MAPK/ERK activation [16]. The activation of oxidation-sensitive MAPKs is generally demonstrated by either a pharmacological or genetic analysis of the kinetics of protein phosphorylation in the presence or absence of

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anti-oxidative treatment [17]. These experimental approaches are acceptable methods for evaluating ROS-mediated MAPK signal transduction.

Chemiluminescent and fluorescent probes are the most commonly used probes for the detection of ROS [18,19]. These probes show high sensitivity, simplicity, and reproducibility, but they lack selectivity because they react with several types of ROS. Dichlorofluorescein (DCF) is currently widely used for detecting H_2O_2 and is suitable for measuring total ROS in living cells or tissues [20–22]. Upon oxidization, the non-fluorescent species DCFH is converted to the fluorescent species DCF, which can be easily detected by flow cytometry as well as fluorescence microscopy. Until now, it has been technically challenging for researchers to simultaneously detect ROS generation and cellular oxidative responses within the same cells. For cellular protein analysis, cells are typically fixed and then subjected to antibody-based immunostaining. However, the effects of these fixatives on DCF-based ROS detection have not been well characterized. This study is aimed at developing a modified fixed staining method to simultaneously detect protein expression in response to oxidative stress and ROS. To this end, we examined cells that received exogenously administered H_2O_2 and cells that underwent endogenous ROS generation following pharmacological and physiological stimulation. For this study, we used fixation-based immunostaining and the cell-permeable free radical sensor 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), which is nonfluorescent until its acetate groups are removed by intracellular esterases and oxidation.

2. Materials and methods

2.1. Reagents

H_2O_2, methanol, acetic acid, formaldehyde, ethanol, and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma–Aldrich (St. Louis, MO) and Calbiochem (San Diego, CA). Antimycin A was purchased from Sigma–Aldrich and dissolved in dimethyl sulfoxide. Rabbit anti-human phospho-SHP2 (Tyr542), SHP2, phospho-ERK (Thr202/Tyr204), ERK, phospho-JNK1/2 (Thr183/Tyr185), JNK1/2, phospho-p38 MAPK (Thr180/Tyr182), and p38 MAPK antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-β-actin antibodies and horseradish peroxidase- or Alexa594-conjugated anti-rabbit IgG were obtained from Chemicon International (Temecula, CA). All drug treatments in cells were assessed for their cytotoxic effects prior to the experiments. Non-cytotoxic dosages were used in this study.

2.2. Cell culture

Human lung adenocarcinoma A549 cells were obtained from ATCC (CCL-185). The human lung adenocarcinoma cell line AS2 was established from ascites that had been generated using PC14PE6 cells (a gift from Isaiah J. Fidler, MD Anderson Cancer Center, Houston, TX) in nude mice [23]. A549 and AS2 cells were routinely grown in plastic dishes in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY) containing L-glutamine and 15 mM HEPES and supplemented with 10% fetal bovine serum (Gibco-BRL), 100 units of penicillin, and 100 μg/ml streptomycin, maintained at 37 °C in 5% CO_2. Immortalized human skin-derived microvascular endothelial cells (HMEC-1) were received from the Centers for Disease Control and Prevention (Atlanta, GA) and cultured in MCDB 131 medium (Gibco-Invitrogen, Karlsruhe, Germany) supplemented with 1× Glutamax I (Invitrogen), 10% fetal calf serum (PAA Laboratories, Coelbe, Germany), 50 ng/ml amphotericin B, and 50 μg/ml gentamicin (PromoCell, Heidelberg, Germany), according to the manufacturer's instructions. Other materials used for cell culture were purchased from Sigma–Aldrich. Under serum starvation conditions, the cells were cultured in serum-free culture medium for 3 or 12 h.

2.3. ROS detection and immunostaining

Intracellular ROS levels were measured using dichlorodihydrofluorescein diacetate oxidation. For the microplate reader-based assay, a microplate reader (SpectraMax 340PC; Molecular Devices, Inc., Sunnyvale, CA) was used to measure the absorbance at 525 nm, and the data were analyzed using Softmax Pro software (Molecular Devices). For flow cytometric analyses, cells were exposed to 10 μM CM-H_2DCFDA (Invitrogen, San Diego, CA) for 5 min. The cells were then analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) using the FL-1 channel (515–545 nm). The mean fluorescence intensity (MFI) was analyzed using CellQuest Pro 4.0.2 software, and quantification was performed using WinMDI 2.8 software (The Scripps Institute, La Jolla, CA). Small cellular debris was excluded by gating on a forward scatter plot. For immunostaining of CM-H_2DCFDA and costaining of phospho-p38 MAPK (Thr180/Tyr182) and phospho-SHP2 (Tyr542), starvation- or H_2O_2-treated and starved cells were stained with CM-H_2DCFDA as described above and subsequently fixed in 10% methanol or 0.1% acetic acid in phosphate-buffered saline (PBS) for 5 min. After washing twice with PBS, the cells were incubated with primary and secondary antibodies, analyzed using a FACSCalibur flow cytometer, and visualized under a laser-scanning confocal microscope (SPII) (Digital Eclipse C1si-ready; Nikon, Tokyo, Japan). DAPI (1:200) was added as a nuclear counter-stain at room temperature for 10 min.

2.4. Western blot analysis

Harvested cells were lysed in a buffer containing 1% Triton X-100, 50 mM Tris (pH 7.5), 10 mM EDTA, 0.02% NaN_3, and a protease inhibitor cocktail (Roche Boehringer MannheimDiagnostics, Mannheim, Germany). Following one freeze–thaw cycle, the cell lysates were centrifuged at 10,000g at 4 °C for 20 min and boiled in sample buffer for 5 min. The proteins were then subjected to SDS–PAGE and transferred to a PVDF membrane (Millipore, Billerica, MA) using a semi-dry electroblotting system. After blocking with 5% skim milk in PBS, the membranes were incubated with primary antibodies (1:1000 dilution) at 4 °C overnight. The membranes were then washed with PBS containing 0.05% Tween 20 and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000 dilution) at room temperature for 1 h. After washing, the membranes were soaked in ECL solution (PerkinElmer Life Sciences Inc., Boston, MA) for 1 min, according to the manufacturer's instructions, and exposed to film (BioMax; Eastman Kodak, Rochester, NY). Other materials used for protein analyses were purchased from Sigma–Aldrich.

2.5. Short-hairpin RNA

Protein expression was downregulated using the lentiviral expression of a short hairpin RNA (shRNA) targeting human SHP2 (shRNA clone ID TRCN0000005003, containing the following shRNA target sequence: 5′-CGCTAAGAGAACTTAAACTTT-3′) and a negative control construct (luciferase shRNA, shLuc). shRNA clones were obtained from the National RNAi Core Facility, Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taipei, Taiwan. Lentiviruses were prepared and cells were infected according to previously described protocols [24]. Briefly, A549 cells were transduced with lentivirus at an appropriate multiplicity of infection in complete growth medium supplemented with

polybrene (Sigma–Aldrich). After transduction for 24 h and puromycin (Calbiochem, San Diego, CA) selection for 3 days, protein expression was analyzed by Western blot.

2.6. Statistical analysis

All values are expressed as the means ± standard deviation (SD). Groups were compared using Student's two-tailed, an unpaired *t*-test, or one-way ANOVA analysis followed by a Dunnett post hoc test, as appropriate. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Differing effects of fixatives on free radical sensors in response to H_2O_2 and on the fluorescent oxidized products of free radical sensors in vitro

An alternative protocol using modified fixed staining methods for the simultaneous detection of different oxidative responses, particularly ROS generation and oxidative stress-associated protein expression, was evaluated in this study. The exogenous administration of H_2O_2 was used as an oxidative stimulant both *in vitro*, in a cell-free system, and *in vivo*, in cultured cells [25]. CM-H_2-DCFDA, a chloromethyl derivative of H_2DCFDA, was used as the free radical sensor. The generation of fluorescent DCF by H_2O_2 oxidation could be accurately ($p < 0.05$) measured using microplate reader-based analysis (Fig. 1A). We next used two experimental models, as summarized in Fig. 1B, to evaluate the effects of several fixatives, including methanol (10% in PBS), acetic acid (0.1% in PBS), formaldehyde (3.7% in PBS), and ethanol (10% in PBS), on CM-H_2-DCFDA in response to H_2O_2 and on the oxidized fluorescent products of CM-H_2DCFDA *in vitro*. In Model A (Fig. 1C), CM-H_2DCFDA was mixed with each fixative for 5 min and subsequently treated with H_2O_2 for another 5 min. In Model B (Fig. 1D), the mixtures of CM-H_2DCFDA and H_2O_2 were prepared using the standard method and then incubated with the fixatives for 5 min. The results showed that fixation with methanol alone had the smallest effect on the H_2O_2-mediated oxidation of CM-H_2DCFDA and on the fluorescence of oxidized DCF *in vitro*. Fixation with acetic acid did not decrease the levels of CM-H_2DCFDA oxidation; however, it appeared to decrease the fluorescence of oxidized DCF *in vitro*. The results also showed that fixation with ethanol significantly inhibited ROS generation.

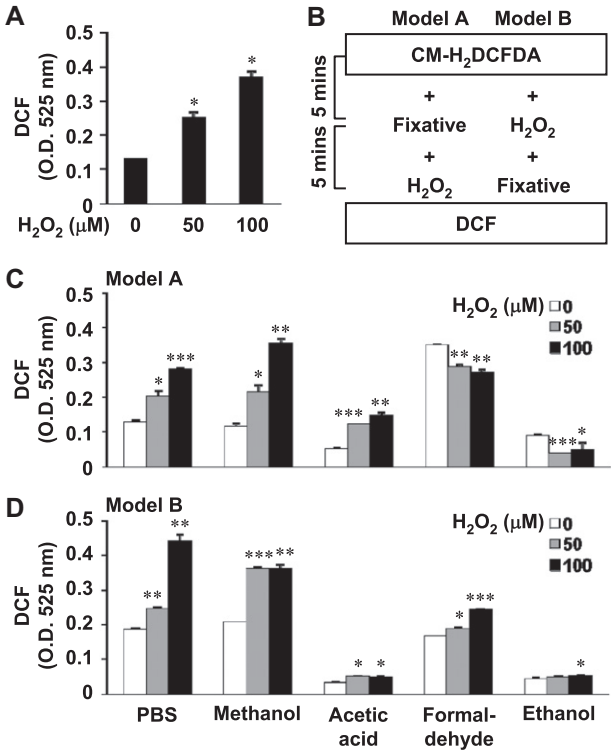

Fig. 1. The effects of fixation on CM-H_2DCFDA oxidation *in vitro*. (A) CM-H_2DCFDA (10 μM) was incubated with the indicated doses of H_2O_2 for 5 min at room temperature. (B) An experimental flowchart of models A and B. (C) For Model A, CM-H_2DCFDA (10 μM) was treated with or without the indicated fixatives, including 10% methanol in PBS, 0.1% acetic acid in PBS, 3.7% formaldehyde in PBS, and 10% ethanol in PBS, for 5 min prior to incubation with H_2O_2 (50 or 100 μM) for 5 min at room temperature. (D) For Model B, mixtures of CM-H_2DCFDA (10 μM) with H_2O_2 (50 or 100 μM) were incubated with the indicated fixatives for 5 min at room temperature. The generation of fluorescent oxidized DCF, shown as the means ± SD optical density (O.D.) measurements from triplicate cultures, was determined using a microplate reader with the absorbance set at 525 nm. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus untreated control.

3.2. Comparison of the effects of fixatives on free radical sensors and their fluorescent oxidized products in response to oxidative stress in vivo

For most biological redox studies, oxidative responses within cells, such as ROS generation, are typically investigated *in vivo* using oxidized DCF staining followed by flow cytometric analysis and/or microscopic observation [21,22]. For experimental Model B, we evaluated the effects of the fixatives, specifically methanol (10% in PBS) and acetic acid (0.1% in PBS), on ROS detection *in vivo* using CM-H_2DCFDA staining followed by flow cytometric analysis. In A549 cells, a similar background level of ROS was observed with or without fixation, as shown in Fig. 2A. A549 (Fig. 2D) and HMEC-1 cells (Fig. 2E) were treated with H_2O_2 (Fig. 2B) or antimycin A (Fig. 2C) (an inhibitor of respiratory complex III of the electron transport system that causes ROS generation [26,27]) or serum-starved [28], and the oxidized DCF generated was measured. Notably, fluorescence was not inhibited by fixation in methanol. To confirm these findings, AS2 cells, human lung adenocarcinoma cells that have high endogenous levels of ROS [23], was performed (Fig. 2F). The results of these experiments demonstrate the potential application of modified fixation in detecting ROS *in vivo*.

3.3. Simultaneous flow cytometric and immunocytochemical detection of ROS and oxidative responses in fixed cells

We successfully created a modified fixed staining method for detecting ROS *in vitro* and *in vivo*. Using fixation-based immunostaining in cells under serum starvation conditions [28], we next evaluated the possibility of simultaneously detecting ROS generation and cellular oxidative responses (such as redox-sensitive protein phosphorylation) within cells [16]. Flow cytometric analysis of DCF generation and p38 MAPK phosphorylation (Thr180/Tyr182) revealed co-staining in the starved HMEC-1 cells fixed with acetic acid (0.1% in PBS) but not in non-starved cells (Fig. 3). Using CM-H_2DCFDA-staining followed by flow cytometric analysis, we characterized the time-dependent production of oxidized DCF by H_2O_2 in A549 cells (Fig. 4A). Western blot analysis showed that treatment with H_2O_2 also resulted in the sequential phosphorylation of SHP2 and SHP2-regulated MAPKs, including ERK, JNK1/2, and p38 MAPK, as demonstrated using a lentiviral-based shRNA approach (Fig. 4B). A549 cells were treated with H_2O_2 for 5 min, and CM-H_2DCFDA was used to detect ROS. The cells were then fixed with methanol (10% in PBS) or acetic acid (0.1% in PBS), followed by immunostaining with phosphorylated SHP2 (Tyr542). Confocal fluorescent microscopic observation revealed co-staining in fixed cells but not in unfixed cells (Fig. 4C). These results dem-

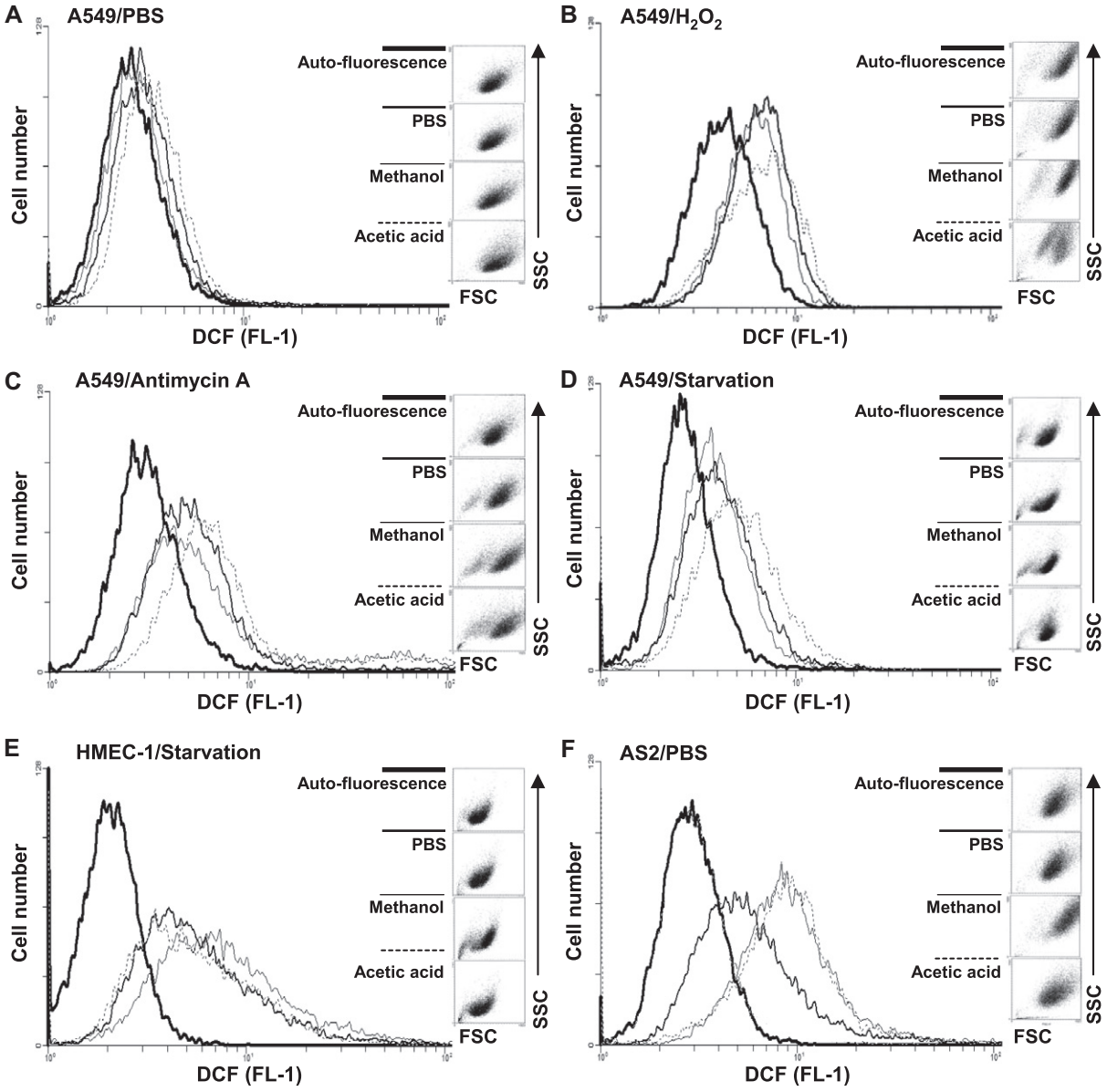

Fig. 2. The effects of fixatives on CM-H_2DCFDA oxidation *in vivo*. Following the protocol of Model B, (A) PBS-, (B) H_2O_2 (100 μM for 30 min)-, (C) antimycin A (5 μM for 30 min), or (D) serum starvation (for 12 h)-treated A549 cells, (E) serum starvation (for 3 h)-treated HMEC-1 cells, and (F) PBS-treated AS2 cells were incubated with CM-H_2DCFDA (10 μM) for 5 min and subsequently incubated with or without the indicated fixatives, including 10% methanol in PBS and 0.1% acetic acid in PBS, for another 5 min at room temperature. Representative flow cytometric histograms (cell number/DCF, FL-1) and dot plots (SSC/FSC) of the auto-fluorescence and generation of fluorescent oxidized DCF were obtained from three individual experiments.

onstrate that a modified fixed staining method can be used to detect ROS and oxidative stress-associated proteins simultaneously.

4. Discussion

Experiments to study the regulatory effects of oxidation on cellular responses typically involve the use of antioxidants and inhibitors of ROS generation [29,30]. However, it is difficult to simultaneously monitor cellular oxidative responses and ROS generation in ROS-generating cells until time course experiments and/or anti-oxidant treatments have been performed. Following ROS generation, oxidized proteins, lipids, and DNA can be analyzed to confirm the induction of intracellular oxidation, often through the use of antibody-based immunostaining [6–8]. However, the simultaneous detection of ROS and oxidative responses, such as protein expression, activation, and post-modification, is not frequently reported because protocols using DCF-based measurements of ROS are generally performed in live-cell systems. In contrast, antibody-based immunostaining techniques usually rely on the use of fixatives such as formaldehyde and acetic acid. The hydrolytic properties of these fixatives may interfere with DCF stability and activity. This study describes a modified fixed staining method to detect oxidative protein expression in cells that have generated ROS upon the exogenous administration of H_2O_2 or in response to pharmacological and physiological stimulation.

Generally, standard immunostaining methods to detect protein expression use fixatives to achieve membrane permeabilization and protein fixation. The results of this study demonstrate a protocol that can be used to simultaneously detect ROS and protein expression. Using an *in vitro* approach, we found that of the fixatives tested, only methanol (10% in PBS) did not affect the

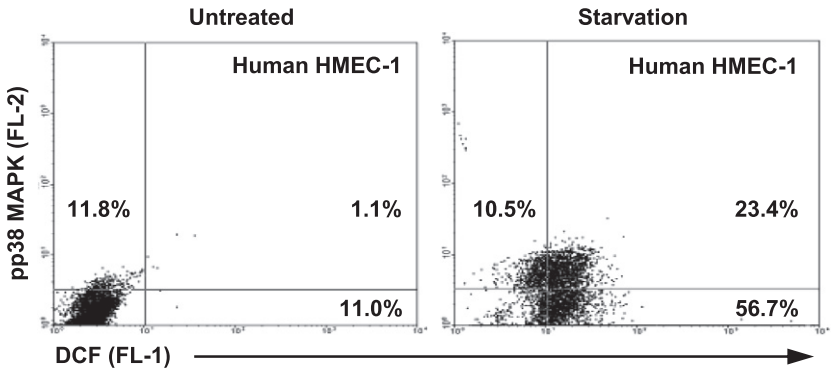

Fig. 3. Flow cytometric analysis of a modified fixed staining method for the simultaneous detection of ROS and oxidative responses. Following the protocol shown in Model B, CM-H_2DCFDA (10 μM) and phosphorylated p38 MAPK (Thr180/Tyr182, *pp38 MAPK*) staining followed by flow cytometric analysis was used to simultaneously determine the presence of ROS and oxidative stress-associated protein phosphorylation in serum-starved (for 3 h) HMEC-1 cells with or without acetic acid (0.1% in PBS) fixation. A representative flow cytometric dot plot (pp38 MAPK, FL-2/DCF, FL-1) chosen from three individual experiments is shown.

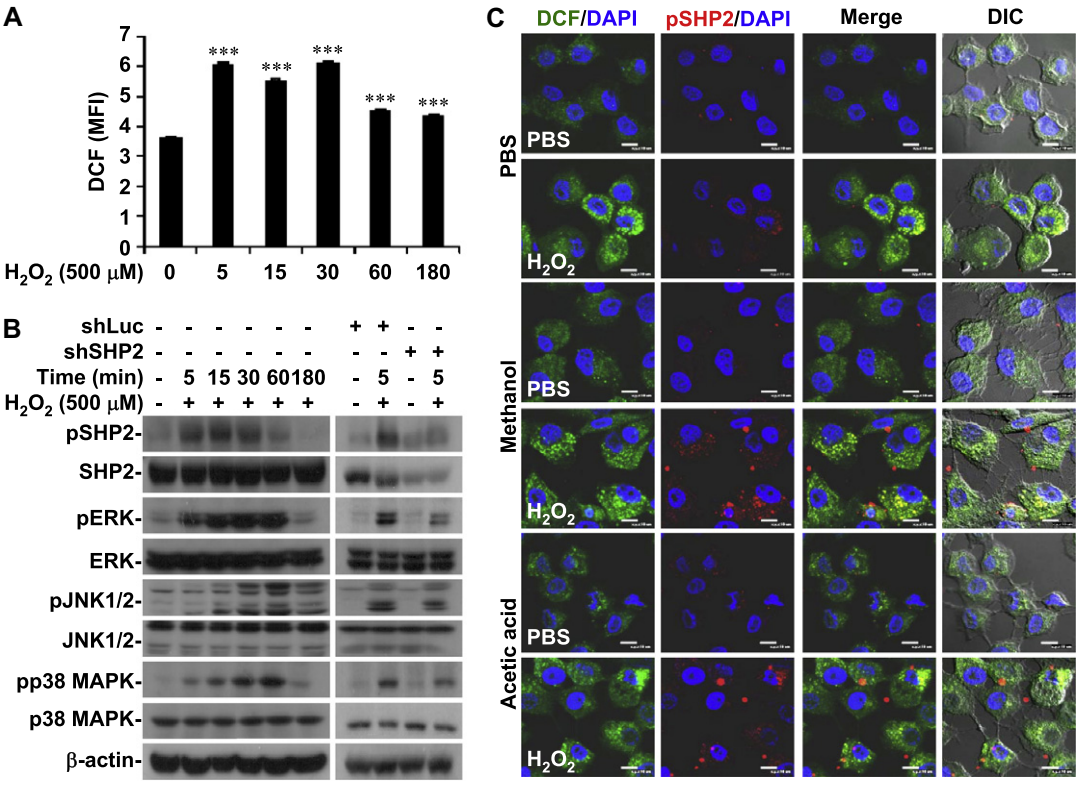

Fig. 4. Immunostaining using the modified fixed staining method to detect ROS and oxidative responses simultaneously. Following the protocol of Model B, (A) flow cytometry and (B) Western blot analyses were performed to detect the generation of fluorescent oxidized DCF (shown as the MFI ± SD of triplicate cultures), and the expression of proteins including SHP2 (Tyr542, *pSHP2*), SHP2, ERK (Thr202/Tyr204, *pERK*), ERK, JNK1/2 (Thr183/Tyr185, *pJNK1/2*), JNK1/2, p38 MAPK (Thr180/Tyr182, *pp38 MAPK*), and p38 MAPK in H_2O_2 (500 μM for the indicated time periods)-treated A549 cells with or without lentiviral-based shRNA transfection targeting of SHP2 (*shSHP2*) and luciferase (*shLuc*). ***$p < 0.001$ versus untreated control. β-Actin was used as an internal control. One representative dataset obtained from three individual experiments is shown. (C) CM-H_2DCFDA and phosphorylated SHP2 (Tyr542, *pSHP2*) staining and subsequent confocal fluorescent microscopic observation were used to determine the simultaneous presence of ROS (*green*) and oxidative stress-associated protein phosphorylation (*red*) (*merge*) in H_2O_2 (500 μM for 5 min)-treated A549 cells that were or were not fixed in methanol (10% in PBS) or acetic acid (0.1% in PBS). Differential interference contrast (*DIC*) imaging shows the location of fluorescent signals within the cells. DAPI (*blue*) was used as a nuclear counterstain. One representative image selected from three individual experiments is shown. The scale bar is 10 μm.

H_2O_2-mediated oxidation of CM-H_2DCFDA (demonstrated in Model A) or the fluorescence of DCF (Model B). Further studies *in vivo* using Model B in H_2O_2-, antimycin A-, and starvation-treated cells also demonstrated that both methanol- and acetic acid-based fixation methods had no effects on the maintenance of DCF fluorescence. These results show for the first time that both endogenously and exogenously generated ROS can be detected in fixed cells. However, consistent with the half-life of DCF in live cells, DCF fluorescence is not maintained after fixation (data not shown). Our analysis of a modified fixed staining method to detect ROS and oxidative responses demonstrates the effectiveness of this protocol.

For immunostaining, double-staining techniques are usually performed to allow the simultaneous detection of two signals in cells. Based on the findings of this study, we developed a method for combination staining using fixation-based immunostaining methods. The activation of p38 MAPK by oxidative signaling has been demonstrated previously [11–13]. Consistent with the find-

ings in starved cells [31], and as previously shown through flow cytometric analysis [21,22], we found that phosphorylated p38 MAPK and ROS can be detected simultaneously in ROS-generating cells. This finding provides direct evidence of the correlation between ROS generation and oxidation-induced p38 MAPK activation in the same cells. The exogenous administration of H_2O_2 is commonly performed to induce oxidative stress signaling [25]. Consistent with previous studies demonstrating that SHP2 mediates the activation of MAPK [14–16], this study confirms the important role of SHP2 upstream of MAPK activation using a lentiviral-based shRNA approach. Furthermore, the modified fixation method in combination with a fluorescent image analysis method, as described previously [28], shows further advantages in that it can be used to verify the co-expression of ROS and active SHP2 (as shown by SHP2 phosphorylation at tyrosine 542), in the early stages of stimulation. Our results strengthen the conclusion that SHP2 is activated by ROS-mediated oxidative stress [14]. We also provide evidence to support the use of a modified method to allow the simultaneous co-staining of ROS and oxidative responses using fixation-based immunostaining. We speculate that this modified method will prove useful for studying redox signaling pathways within the same cells, thereby providing a useful tool for future biomedical research on the cell cycle, growth, differentiation, and apoptosis.

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