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Cell lysis with dimethyl sulphoxide produces stable homogeneous solutions in the dichlorofluorescein oxidative stress assay

GUQI WANG, YU GONG, FRANK J. BURCZYNSKI, & BRIAN B. HASINOFF

Faculty of Pharmacy, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada

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Abstract
The oxidation of 2′,7′-dichlorodihydrofluorescein (2′,7′-dichlorofluorescin, DCFH) to a fluorescent product, 2′,7′-dichlorofluorescein (DCF), is commonly used to quantitatively measure oxidative stress in cells using a fluorescence microplate reader. However, many cell lines tend to grow non-uniformly in the wells. This non-uniform distribution results in a high degree of variability in the fluorescence signal and decreases the precision of the method. Also, samples treated in large culture plates, dishes or flasks cannot be assayed directly in fluorescence microplate readers. This study reports an improved DCF assay method that lyses cells with DMSO/PBS (90% dimethyl sulphoxide/10% phosphate buffered saline). Oxidative stress was induced with either hydrogen peroxide or an hypoxia-reoxygenation treatment. Cell lysis with DMSO/PBS resulted in highly stable fluorescence signals in comparison to Triton X-100/PBS lysed cells. The precision of DCF fluorescence measurements of DMSO/PBS lysed cells was much better than for attached cells measured directly in 96-well plates. While DCF fluorescence in PBS was strongly quenched by albumin, no quenching occurred in DMSO/PBS. In conclusion this study describes a more convenient and accurate method for measuring cellular oxidative stress that also makes it possible to assay cells treated in large culture plates.

Keywords: *Oxidative damage, fluorescence, reactive oxygen species (ROS), dichlorofluorescin, dimethyl sulphoxide, dichlorofluorescein*

Abbreviations: *DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DCFH, 2′,7′-dichlorodihydrofluorescein; DCF, 2′,7′-dichlorofluorescein; 90% DMSO/10% PBS, 90% DMSO/10% PBS (v/v); DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; FBS, foetal bovine serum; LDH, lactate dehydrogenase; L-FABP, liver fatty acid binding protein; PBS, phosphate buffered saline; ROS, reactive oxygen species; SEM, standard error of the mean*

Introduction

The oxidation of 2′,7′-dichlorodihydrofluorescein (2′,7′-dichlorofluorescin, DCFH) to a fluorescent product, DCF, is commonly used as a highly sensitive fluorometric method to assay ROS formation in cells. Since DCFH is not cell permeable, its diacetate ester, DCFH-DA, which is more cell-permeable, is used. DCFH-DA is deacetylated to DCFH by intracellular esterases when loaded into cells. Oxidation of DCFH produces DCF that can easily be detected by its strong fluorescence at 525 nm. The DCF fluorescence assay is, however, not a direct assay of H_2O_2, nitric oxide, lipid peroxides, singlet O_2 or $O_2^{\bullet -}$ because these species react very slowly with DCFH in the absence of ferrous iron [1–3]. However, peroxyl, alkoxyl, $NO_2^{\bullet}$, carbonate ($CO_3^{\bullet -}$) and $OH^{\bullet}$ radicals, as well as peroxynitrite, can quickly oxidize DCFH to DCF [1,4–8]. Therefore, the DCF assay can only detect generalized cellular oxidative

Correspondence: Brian B. Hasinoff, Faculty of Pharmacy, University of Manitoba, 50 Sifton Road, Winnipeg, Manitoba R3T 2N2, Canada. Tel: (204) 474-8325. Fax: (204) 474-7617. Email: b_hasinoff@umanitoba.ca

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stress efficiently when peroxides are decomposed by iron/heme proteins or other radicals. Nevertheless, when properly used, DCFH oxidation is a powerful probe for measuring oxidative stress. The formation of ROS and DCF is highly linearly correlated [9–11].

Caution should be taken in apoptosis studies using DCF, because cytochrome *c* is a powerful catalyst of DCFH oxidation and the cytochrome *c* released from mitochondria during apoptosis may produce a significant DCF signal without any change in cellular peroxide levels [12]. Some compounds, like pyocyanin, mitoxantrone and ametantrone can directly oxidize DCFH and result in an artificial measure of oxidative stress [13]. In some cases a decreased DCF fluorescence signal may not be due to reduced oxidative stress, but to quenching of the fluorophore fluorescence. Native or modified albumin suppresses the fluorescence of oxidized DCF by binding to the protein hydrophobic pocket in domains II and III [14]. A fluorescence microplate reader affords an easy and efficient method to measure DCF fluorescence produced by oxidative stress and for the testing of compounds that may prevent oxidative stress in cells [15,16]. However, there are obstacles in achieving accurate results in the DCF assay because many cell lines tend to grow in a non-uniform distribution in the wells. This non-uniform distribution may result in a high degree of variability in the fluorescence signal, thus decreasing the precision of the method. Also cells treated in large culture plates, dishes or flasks cannot be assayed directly in a fluorescence microplate reader. Moreover, the formed DCF may diffuse out of the cells [9], resulting in light emission from the medium as well as from the cells. In this study, we report an improved method to quantify oxidative stress in cells using the DCF assay by lysing the cells with a mixture of DMSO and aqueous PBS. This method produced highly stable, homogenous solutions of DCF that were unaffected by albumin.

Materials and methods

DCFH-DA, DCF and other chemicals were purchased from Sigma-Aldrich (Oakville, Canada). Adherent Chang liver and rat hepatoma 1548 cells and K562 suspension cells were obtained from the ATCC (Manassas, VA). Chang liver cells over-expressing liver fatty acid binding protein (L-FABP), which we previously showed to have an increased cellular antioxidant capacity, were also used [17]. On the day of the experiment, a 10 mm stock solution of DCFH-DA was prepared in ethanol and stored at -20°C until used. This stock was diluted to 100 μM with serum-free DMEM medium (to avoid binding to albumin) prior to each experiment. A FLUOstar Galaxy plate reader (BMG, Durham, NC) thermostated at 25°C with 485 nm excitation and 520 nm emission wavelengths was used to measure DCF fluorescence. A *t*-test was used to determine significance. Error bars shown are standard errors of the means. When the percentage composition of the DMSO/PBS mixtures are given they are on a v/v basis.

Hydrogen peroxide-induced cellular oxidative stress

Chang cells or 1548 cells were cultured in 96-well black assay plates (Costar #3603, Corning, NY) at a density of 25 000 cells/well. After 8 h incubation in DMEM-10% FBS, cultures were washed twice with PBS to remove endogenous esterase activity contained in the FBS. Cells were loaded with DCFH-DA by treating them with 100 μl of 100 μM DCFH-DA for 30 min at 37°C. Extracellular DCFH-DA was removed by washing twice with PBS. To induce cellular oxidative stress the attached cells were incubated with 100 μl of 400 μM H_2O_2 in PBS containing Ca^{2+} (0.9 mm) and Mg^{2+} (0.5 mm) for 15 min at room temperature in the dark. The cells were washed with PBS and the supernatant was removed. The cells were then lysed by adding 50 μl of 90% DMSO/10% PBS for 10 min in the dark at room temperature with shaking. The fluorescence intensity in each well was then measured in the fluorescence plate reader.

For K562 suspension cells, 4 ml of the cell suspension were loaded into a centrifuge tube at a cell density of 1.25 million cells/ml and centrifuged at 400 *g* for 6 min. The supernatant was removed and 1 ml of 100 μM DCFH-DA was added to resuspend the cells. The cells were incubated for 30 min at 37°C with gentle shaking. After washing the cells twice with PBS to remove extracellular DCFH-DA, cellular oxidative stress was induced by incubating the cell suspension in 1 ml of 400 μM H_2O_2 in PBS for 15 min at room temperature in the dark with gentle shaking. After the H_2O_2 supernatant solution was removed after centrifugation at 400 *g* for 6 min, the cell pellets were lysed by adding 0.5 ml of 90% DMSO/10% PBS and shaken (150 rpm for 5 min). The K562 cell lysates were centrifuged at 3000 *g* for 5 min to precipitate cell debris. The cell lysates were then added to a 96-well black assay plate (120 μl/well) and the DCF fluorescence was measured for various times. The H_2O_2 treatment was omitted in the control.

Hypoxia-reoxygenation induced oxidative stress and LDH assay

The hypoxia-reoxygenation experiments were done as we previously described [17,18]. Adherent Chang cells were seeded in 35-mm culture dishes at a cell density of one million cells/dish. After an 8 h incubation in DMEM-10% FBS, the culture medium was replaced three times with 1 ml DMEM-0% FBS to minimize background LDH levels. Culture dishes

were placed in the hypoxia apparatus and made hypoxic by passing 95% N_2/5% CO_2 over the surface of the liquid for 3 h at a flow rate of 80 ml/min. Cells were subsequently reoxygenated with medical-grade 95% O_2/5% CO_2 for 3 h at the same flow rate. To maximize gas exchange during hypoxia, the hypoxia apparatus was gently shaken. At the end of the hypoxia-reoxygenation treatment, a 100 μl sample of the supernatant was collected for LDH assay and centrifuged for 5 min at 500 *g* to remove any floating cells. Samples were stored at −80°C until the LDH was assayed. After the supernatant was sampled, the cells were treated with 1 ml of 100 μM DCFH-DA for 30 min in a CO_2 incubator. Plates were then washed twice with PBS, the supernatant was removed and the cultures were lysed with 0.5 ml 90% DMSO/10% PBS for 10 min at room temperature in the dark. Lysate (200 μl) was collected and the DCF fluorescence was immediately measured in a 96-well plate.

LDH is a cytosolic enzyme present in all mammalian cells and release of LDH into the culture medium is a measure of cell membrane integrity and cell viability. LDH activity was also measured using a spectrophotometric kinetic assay as we previously described [17] in order to compare it to the DCF assay.

Results and discussion

The effect of DMSO/PBS composition on DCF fluorescence and a comparison of 100% DMSO and 90% DMSO/10% PBS lysis on H_2O_2-treated cells

The effect on DCF fluorescence of varying the proportion of DMSO in the lysing solution was studied in order to determine the sensitivity to solvent composition. Because oxidized DCF has an ionizable carboxylic acid group and a phenol group [4], the pH and the composition of the solvent may affect DCF fluorescence. In a comprehensive study the pK_a values of DCFH, its free radical intermediates and oxidized DCF were measured [19]. DCF displays three measurable proton equilibria with pK_a values of 0.85, 3.5 and 5.19 [19]. However, under the conditions of this study in PBS (pH 7.4) the pH was sufficiently higher than the highest pK_a so that the fluorescence emission spectrum should be insensitive to small changes in pH. As shown in Figure 1A, increasing the proportion of PBS in DMSO-PBS mixtures increased DCF fluorescence. The DCF fluorescence was linearly related to DCF concentration in the low nanomolar to micromolar concentration range for all but 100% DMSO. The lack of linearity in Figure 1A at higher DCF concentrations may be due to an inner filter effect due the solution absorbing some of the light emitted. Lysis of H_2O_2-treated 1548 cells with 90% DMSO/10% PBS and with 100% DMSO are compared in Figure 1B. A 23% larger fluorescence signal was seen using 90% DMSO/10% PBS compared to 100% DMSO. It was for this reason and the desire to maintain the pH constant that 90% DMSO/10% PBS was used in subsequent experiments. The 90% DMSO/10% PBS mixture was chosen for the cell studies as this composition not only maintained a good fluorescence signal (Figure 1A) but it also contained a high enough percentage of DMSO to efficiently lyse cells, prevent binding to albumin and inhibit cellular enzymes that could oxidize DCF.

90% DMSO/10% PBS prevents quenching of DCF fluorescence by albumin

Binding of DCF to albumin leads to fluorescence quenching of DCF [14], which may be problematic in the DCF assay. In order to determine if quenching of DCF fluorescence could be prevented by 90%

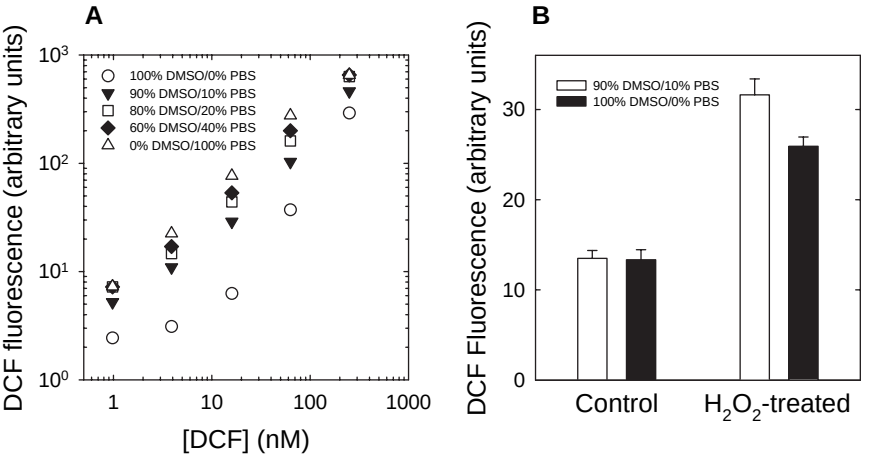

Figure 1. Effect of solvent composition on DCF fluorescence in DMSO-PBS solutions and a comparison of H_2O_2-induced DCF fluorescence in 1548 cells lysed with 100% DMSO alone and 90% DMSO/10% PBS. (A) Change in fluorescence with DCF concentration in DMSO-PBS solutions of various proportions. (B) H_2O_2-induced oxidative stress in 1548 cells. Attached cells were lysed with either 100% DMSO or 90% DMSO/10%PBS. The DCF fluorescence in the 90% DMSO/10% PBS lysate was 23% higher than in the 100% DMSO lysate ($n = 8$, $p < 0.001$).

DMSO/10% PBS, DCF (20 nm) fluorescence was measured in the presence of various concentrations of fatty acid-free albumin (Figure 2). The fluorescence of DCF in PBS solution was significantly quenched by 10 μM and 100 μM albumin ($p < 0.001$, $n = 6$). However, the fluorescence of DCF (20 nm) in 90% DMSO/10% PBS solution was unchanged by the addition of either 10 μM or 100 μM albumin ($p > 0.05$) which are concentrations that are typically used in culture media. Thus, 90% DMSO/10% PBS inhibits the quenching of DCF by albumin, likely by preventing its binding, and avoids the problem of fluorescence quenching by serum proteins.

DCF fluorescence was stable in 90% DMSO/10% PBS cell lysates, but not in Triton X-100/PBS lysates

K562 cells are human erythroleukemic cells that normally grow in suspension. We studied cellular oxidative stress of K562 cells that had been treated with 400 μM H_2O_2 for 15 min at room temperature; 90% DMSO/10% PBS cell lysates (200 μl) from H_2O_2-treated cells, control cells and a 2 nm DCF solution in 90% DMSO/10% PBS for comparison, respectively, were added to a 96-well plate and measured in the fluorescence plate reader for times up to 120 min. As shown in Figure 3A the DCF fluorescence of 90% DMSO/10% PBS lysates from H_2O_2-treated, control cells and oxidized DCF in 90% DMSO/10% PBS solution were highly stable over 2 h (no significant difference at all times, $p > 0.05$, $n = 8$). However, when K562 cells were lysed with 0.5% (v/v) Triton X-100 in PBS the DCF fluorescence from the H_2O_2-treated cells and control cells slowly increased with time (Figure 3B). In fact after 100 min, in the case of H_2O_2-treated K562 cells, the DCF fluorescence increased to nearly that of the untreated control cell lysates. Similar trends were observed for the Triton X-100 lysate of the 1548 adherent cells (Figure 3B). Ongoing enzymatic oxidation of DCFH to DCF has been implicated in this phenomenon [20]. Triton X-100 is a mild and relatively non-denaturing non-ionic detergent that solubilizes membrane and other proteins while maintaining enzyme function. Lysis with 90% DMSO/10% PBS likely stops the build-up of DCF fluorescence by inhibiting cellular enzyme activities at the stage of cell lysis. The results of Figure 3 show that the DCF fluorescence signal was much more stable after cell lysis with 90% DMSO/10% PBS compared to Triton X-100/PBS. As shown in Figure 3B, DCF alone is even less stable in Triton X-100/PBS than 90% DMSO/10% PBS.

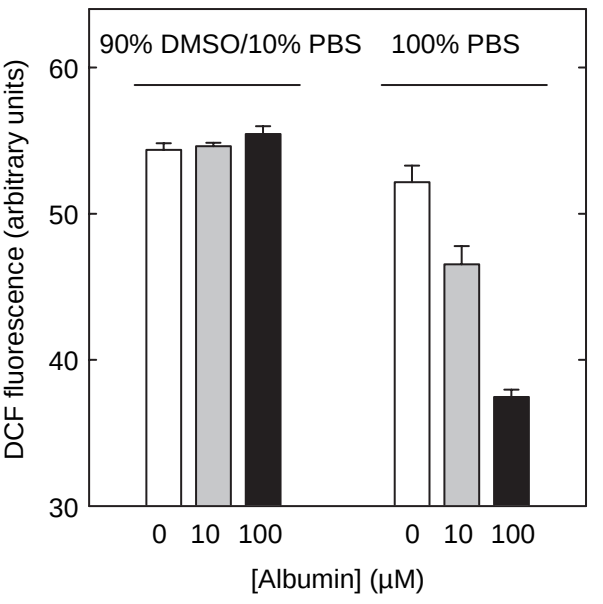

Figure 2. Comparison of albumin-induced quenching of DCF (20 nm) fluorescence in 90% DMSO/10% PBS and in PBS. The DCF fluorescence in PBS was significantly quenched by both 10 and 100 μM albumin ($p < 0.001$, $n = 6$). The fluorescence of DCF in 90% DMSO/10% PBS was not significantly changed by either 10 or 100 μM albumin ($p > 0.05$).

The DCF fluorescence of the 90% DMSO/10% PBS lysates in the treated group was significantly greater than the control at all times for both K562 suspension cells and 1548 attached cells (Figure 3A). Additionally, the factor by which H_2O_2 treatment increased the relative DCF fluorescence was larger for the 90% DMSO/10% PBS treatment than for the Triton X-100/PBS treatment. This may have been due to the fluorescence for the control after Triton X-100/PBS treatment having increased at a more rapid rate than for the H_2O_2 treatment. These results indicate that the 90% DMSO/10% PBS treatment produced a larger relative signal change and a more stable signal in H_2O_2-treated cells than Triton X-100/PBS treatment and thus was a more sensitive assay.

Comparison of H_2O_2-induced DCF fluorescence measured directly in attached cells and in 90% DMSO/10% PBS lysates

Cellular ROS induced by H_2O_2 results in an increase of DCF fluorescence in DCFH-DA loaded cells that is directly proportional to the concentration of H_2O_2 [4]. Experiments were designed in order to compare H_2O_2-induced DCF fluorescence directly in attached cells in a microplate with that obtained after 90% DMSO/10% PBS lysis. Rat hepatoma 1548 cells and L-FABP over-expressing Chang liver cells [17] were used in these experiments. DCF fluorescence was first measured directly on the attached cells. The attached cells were then lysed with 90% DMSO/10% PBS and the DCF fluorescence was measured again. As shown in Figure 4A, 90% DMSO/10% PBS lysis reduced the relative standard error of the fluorescence signal from 9% to 6% in the H_2O_2-induced oxidative stress assay in 1548 cells. This likely occurred because 90% DMSO/10% PBS produced a homogeneous DCF solution. In H_2O_2-induced

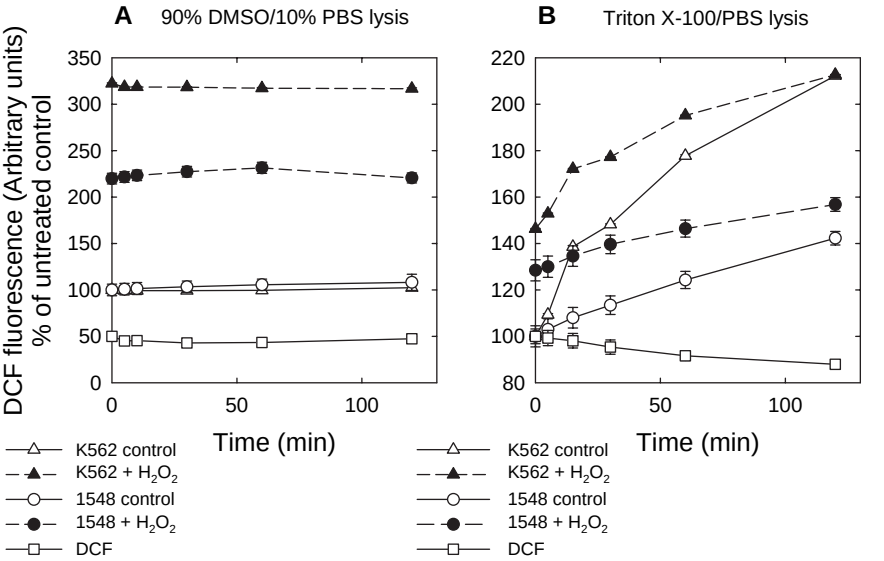

Figure 3. Comparison of the stability of the DCF fluorescence signal in H_2O_2-treated K562 suspension cells and 1548 attached cells lysed with 90% DMSO/10% PBS and with Triton X-100/PBS. (A) Oxidative stress in K562 suspension cells and 1548 attached cells was induced by incubating cells with 400 μM H_2O_2 after being loaded with DCFH-DA. The data is expressed as a percentage relative to the untreated control cells (100%) at time zero ($n=8$). For clarity, the fluorescence of DCF alone was normalized to a value of 50% at time zero. The DCF fluorescence in each group in 90% DMSO/10% PBS was not significantly changed over the whole time ($p>0.05$, $n=8$). The 1548 cells were assayed directly after lysis in the 96-well plate and the K562 cells after lysis of the cell pellet. (B) The K562 cells and the 1548 cells were treated as described above except that the cells were lysed with 0.5% (v/v) Triton X-100 in PBS. The data is presented as a percentage relative to untreated control cells (100%) at time zero as above. Fluorescence signals of both control and H_2O_2-treated cells increased after Triton X-100/PBS lysis, but not after 90% DMSO/10% PBS lysis. The fluorescence of DCF (20 nm) alone in Triton X-100/PBS was normalized to a value of 100% at time zero. Where error bars are not seen they are smaller than the symbol size.

oxidative stress measurements on the two Chang cell lines, the 90% DMSO/10% PBS treatment resulted in a decrease of the relative standard error from 5% to 2% in the low L-FABP expressing cells, while for high L-FABP expressing cells the relative standard error was decreased from 5% to 1%. In fact, the precision of the 90% DMSO/10% PBS method was so improved over the direct DCF measurement that while the decrease in DCF fluorescence achieved significance ($p<0.005$, $n=6$) for the 90% DMSO/10% PBS treatment, that for DCF fluorescence measured directly on attached cells did not. These results also confirm that L-FABP plays a protective role against cellular oxidative stress as we already described [17]. Although some new fluorescence plate readers can perform well scanning by measuring multiple data points in different areas of the well to reduce data variation, 90% DMSO/10% PBS lysis is a convenient method for single point measurements in the well centres that is used for most plate readers.

Hypoxia-reoxygenation induced DCF fluorescence measurement for the samples from large culture dishes

Because multiwell plates are not convenient for the measurement of oxidative stress after hypoxia-reoxygenation due to requirements for efficient gas exchange, hypoxia-reoxygenation experiments using 90% DMSO/10% PBS lysis were conducted on Chang liver cells expressing high and low levels of L-FABP. In this study, cells were cultured in 35-mm tissue culture dishes and subjected to 3 h of hypoxia and 3 h of reoxygenation as previously described [17,18]. The purpose of these particular experiments was to compare the improved DCF assay with the commonly used LDH assay. The cell supernatants were sampled for the assay of LDH activity and the cells were then lysed with 90% DMSO/10% PBS for measurement of DCF fluorescence. The increase in cellular DCF fluorescence induced by hypoxia-reoxygenation treatment is shown in Figure 4C. For comparison, the cell damage caused by the hypoxia-reoxygenation treatment was also determined with the LDH assay (Figure 4D). The results of the increased DCF fluorescence for low L-FABP (liver fatty acid binding protein) expressing Chang cells from DMSO lysates were consistent with the results from the LDH assay. In the usual DCF assay, cells are pre-loaded with DCFH-DA prior to the induction of oxidative stress. However, in the case of a long induction time for oxidative stress, such as that occurring after hypoxia-reoxygenation treatment or after bacterial infection [21], the unreacted DCFH-DA, DCFH and DCF may diffuse out and react in the medium [14,22]. This study and our previous work [21] showed that the DCF assay could be used when DCFH-DA was loaded in cells directly after induction of oxidative stress. These results indicate that oxidative stress was an ongoing process even after the end of the reoxygenation period.

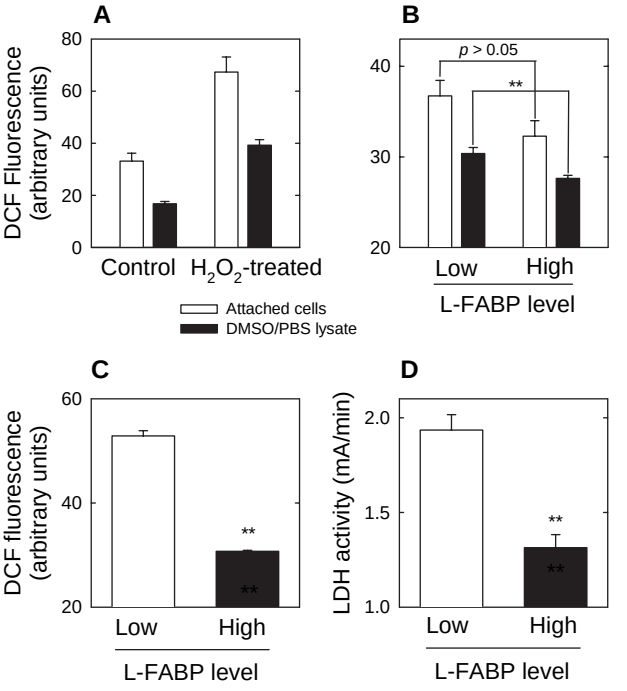

Figure 4. Comparison of H_2O_2-induced DCF fluorescence measured directly in attached 1548 and Chang cells and in 90% DMSO/10% PBS lysates. (A) H_2O_2-induced DCF fluorescence measured directly in 1548 attached cells and after 90% DMSO/10% PBS lysis are compared. The relative errors are smaller for the 90% DMSO/10% PBS lysed cells (relative SEM of 9% for direct measurement vs 5% for 90% DMSO/10% PBS lysis). (B) H_2O_2-induced DCF fluorescence measured directly in attached Chang liver cells (expressing low and high levels of L-FABP) after 90% DMSO/10% PBS lysis are compared. The relative errors are smaller for the 90% DMSO/10% PBS lysed cells. For low L-FABP the relative SEM is 5% for direct measurement vs 2% for 90% DMSO/10% PBS lysis and for high L-FABP the relative SEM is 5% vs 1%. While the difference in the direct measurements on the attached cells between low and high L-FABP levels was not significant ($p > 0.05$), the difference between the 90% DMSO/10% PBS lysed cells was. $^{**}p < 0.005$ ($n = 6$). (C) Comparison of DCF fluorescence and LDH release in Chang liver cells expressing low and high levels of L-FABP subjected to hypoxia-reoxygenation in 35-mm tissue culture dishes. After 3 h of hypoxia and 3 h of reoxygenation the cells were loaded with DCFH-DA for 30 min, washed and then lysed with 90% DMSO/10% PBS. There was a significant difference in fluorescence between cells expressing low and high L-FABP levels, $^{**}p < 0.01$ ($n = 4$). (D) The supernatants were also measured for LDH release in the same experiments. The relative SEM values are much smaller for the DCF measurements than for the LDH measurements. There was a significant difference in LDH release between cells expressing low and high L-FABP levels, $^{**}p < 0.01$ ($n = 4$).

DMSO is a commonly used aprotic polar solvent that dissolves both polar and non-polar compounds. It readily penetrates cell membranes and increases the cross-membrane transport of non-ionized molecules. Treatment with solutions containing high concentrations of DMSO likely results in the loss of cell integrity and even cell lysis. Microscopic examination showed that neither K562 nor Chang cells were completely lysed by 90% DMSO/10% PBS (data not shown). However, epifluorescence microscopy showed that the intracellularly-formed oxidized DCF was evenly distributed in the solution after 90% DMSO/10% PBS treatment (data not shown) which indicates that the membrane was sufficiently permeabilized to allow the DCF to leave.

Even though oxidation of intracellular DCFH to DCF may be affected by many factors and may not always be a measure of ROS formation, it nonetheless remains a useful and widely used probe of ROS production in cells. Cell lysis with 90% DMSO/10% PBS and the resulting homogenization and stability of the DCF fluorescence signal clearly results in a more precise and convenient measure of DCFH oxidation to DCF than other methods commonly used. 90% DMSO/10% PBS lysis also allowed for the assay of cells not cultured in multiwell microplates and eliminated the quenching effect of albumin on DCF fluorescence seen in PBS.

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