Glutathionylation of p65NF-κB correlates with proliferating/apoptotic hepatoma cells exposed to pro- and anti-oxidants

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Abstract. Oxidative stress influences a variety of regulatory proteins, including nuclear factor-κB (NF-κB). NF-κB is critical for maintaining the proliferation/apoptosis balance in hepatocytes. In this study we investigated the causal links between glutathione, NF-κB and hepatocyte damage. HepG2 and 3B cells were exposed to different doses of H_2O_2 or N-acetyl-cysteine (NAC) and the proliferation/apoptosis rate, glutathione forms, and p65NF-κB glutathionylation and activity were analysed. Our results demonstrate that H_2O_2 stopped proliferative response at low doses, but induced apoptosis only at high doses. In contrast, NAC exerted, proportionally to its concentration, a dual role simultaneously increasing both proliferation and apoptosis. Interestingly, the levels of protein-bound glutathione were increased by H_2O_2 and decreased by NAC. Moreover, the antibody recognizing the glutathionylated proteins co-precipitated and -localized with the cytoplasmic inactive form of p65NF-κB in H_2O_2- and NAC-treated cells, even when, in 1 mM NAC-treated cells, a part of p65 was glutathione-free and localized into the nucleus. Apoptotic cells were characterised principally by a cytoskeletal staining of glutathionylation and retention of NF-κB in the cytoplasmic region; whereas in proliferating cells, glutathionylated proteins were concentrated into the perinuclear region and p65NF-κB was traslocated into the nucleus. While cytoplasmic NF-κB retention correlated well with an increased apoptotic rate, a greater expression of this protein was observed in association with the NAC-dependent. In conclusion, our findings suggest that glutathionylation inhibits NF-κB activity causing reduced hepatocyte survival, which is common in several liver diseases.

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Introduction

The tripeptide L-γ-glutamyl-L-cysteinyl-glycine, or glutathione, a ubiquitous thiol, plays a major role in maintaining cellular redox state and regulating signalling pathways more commonly altered by oxidative stress (1-3). The reduced glutathione (GSH) and its oxidized form (GSSG) represent the major cellular redox buffer, therefore the ratio GSH/GSSG is a good indicator for the redox environment of the cell. In fact, a decrease in this ratio is indicative of an increase in oxidative stress (4-6).

Glutathione participates in many intracellular reactions playing different roles in cell physiology and pathophysiology (7-10). Glutathione reactions include, its direct and indirect anti-oxidant action; its role as substrate to produce radicals; its conjugation with NO to form *S*-nitroso-glutathione adducts, its reaction with various electrophiles, physiological metabolites (e.g., estrogens, melanins, prostaglandins, and leukotrienes), and xenobiotics (e.g., bromobenzene and acetaminophen) to form mercapturates; its ability to convert formaldehyde to *S*-formyl-glutathione; and finally, its ability to form mixed disulfides with protein sulfhydryl groups (protein glutathionylation) (11-14). Glutathionylation was shown to regulate, either positively or negatively, a variety of regulatory, structural, and metabolic proteins deeply influencing some important biological processes, including proliferation, apoptosis, differentiation and senescence (3,6).

Recent studies demonstrated that glutathionylation is a physiologically relevant mechanism for controlling the magnitude of activation of the nuclear transcription factors and proteins which are strongly associated with apoptotic response and survival of the cell after an imbalance of redox state, including p53 tumor suppressor protein, p65-subunit of nuclear factor-κB (NF-κB) and caspase-3 (15-17).

It is well known that oxidative stress is involved in the pathogenesis and progression of different liver diseases, such as alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD), fibrosis and cirrhosis. The pathogenesis both of ALD and NAFLD has not been yet elucidated, however,

common molecular mechanisms partly explain the development of progressive liver damage characterizing these diseases (18). The available evidence suggests that, by favouring mitochondrial permeability transition, oxidative stress promotes hepatocyte necrosis and/or apoptosis. Moreover, oxidative mechanisms contribute to liver fibrosis by triggering the release of pro-fibrotic cytokines and collagen gene expression in hepatic stellate cells (19-23). In addition, it is relevant that protein glutathionylation, often altered during oxidative stress-dependent hepatic damage, plays a pivotal role in the pathogenesis of several liver diseases, such as NAFLD (24,25). However, the specific role of protein glutathionylation after alteration of redox state is still unknown.

In this study we investigated the possible role of protein glutathionylation in regulating the cellular response of an *in vitro* model of hepatic cells (two hepatoma cell lines, HepG2 and 3B) after exposure to different concentrations of pro- or anti-oxidant agents. We analysed firstly, proliferation and apoptosis rates in hepatoma cells treated with progressive growing doses of H_2O_2 or N-acetylcysteine (NAC); secondly, the intracellular GSH/GSSG ratio, the level of glutathionylated proteins after the different treatments; finally, the p65NF-κB glutathionylation and nuclear translocation in response to pro- and anti-oxidant agents.

Our data suggest that p65NF-κB glutathionylation is a critical factor in regulating the balance between proliferation and apoptosis as a consequence of the alteration of the hepatocyte redox state.

Materials and methods

Cell culture and treatments. HepG2 and 3B cell lines, purchased from American Type Culture Collection (ATCC; Rockville, MD, USA) are two human hepatoma cell lines often used to perform studies on hepatic cell homeostasis. Cells were grown, as a monolayer, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin G and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37˚C. The cells were cultured at 90% confluence and then $1.5x10^6$ were seeded onto two 10-cm culture dishes to perform the experiments. Stock of cells were routinely frozen and stored in liquid N2.

Different concentrations of H_2O_2 (50, 100, 150, 200 and 500 μM) and NAC (1, 2, 4, 5 and 6 mM) were used to incubate cells at 37˚C for 30 min and 1 h, respectively. Blank cells (non-treated) received an equivalent amount of DMSO (the final concentration of DMSO did not exceed 0.1%). After 4, 24 and 48 h of treatment, cells were harvested and collected for Western blotting.

Determination of cell growth. Cell growth was evaluated by the Trypan Blue dye exclusion assay. Treated and non-treated cells, cultured in a 6-well dish ($5x10^5$), were trypsinized and diluted followed by staining with 0.4% (w/v) Trypan Blue (Invitrogen, Carlsbad, CA, USA). The number of cells excluding or incorporating Trypan Blue were counted under a light microscope (Motic AE21 Microscope, Nanovision Srl, Italy). All cells excluded from the staining with respect to the total were counted at different time points (0 and 48 h) and the resulting number was taken as an index of the growth rate.

Determination of cell proliferation by measurement of BrdU incorporation. The incorporation of BrdU was used as a measure of DNA synthesis in HepG2 and 3B cell lines. Cells were cultured in a 96-well plate at the indicated time points, and then analysed by a colorimetric immunoassay for the quantification of cell proliferation. We used a Cell Proliferation ELISA BrdU kit, according to the suggested protocol (Roche, Indianapolis, IN, USA). Quantitative data of the analysis of BrdU incorporation were converted in units of induction with respect to untreated samples considered as 1.

Determination of cell apoptosis. Cell death was characterized by a method which detects mono- and oligonucleosomes (cytoplasmic histone associated DNA fragments), which provide both a qualitative and quantitative measure of apoptotic cells. It is based on an ELISA kit (Roche) that was used according to the supplier's instructions. The number of apoptotic cells was transformed in units of induction with respect to untreated samples considered as 1.

HPLC equipment and conditions to analyze various forms of glutathione. The cells (differently treated) were sonicated three times for 2 sec in 0.1 ml of 0.1 M potassium phosphate buffer (pH 7.2). After sonication (Sonics Vibra Cell, Sonics & Material Inc., Newtown, CT, USA), levels of total (GSH Tot), reduced glutathione (GSH red), oxidized (GSSG) and protein-bound (ProSSG) glutathione were analyzed by HPLC. HPLC equipment and conditions to analyse various forms of glutathione have been reported (8,9).

Immunofluorescence analysis. For immunofluorescent microscopy, cells were seeded and grown in a 4-well chamber slide (Nunc; Naperville, IL, USA). After already described treatments, cells were fixed in -20˚C methanol/acetone (1:1) for 10 min. Briefly, cells were incubated for 30 min with a blocking buffer containing 1% goat serum and 1% BSA in PBS, and then, incubated overnight with a mouse anti-GSH (1:80) purchased from Virogen (Watertown, MA, USA) recognizing glutathionylated proteins alternatively coupled with rabbit anti-p65 (1:80; Santa Cruz Biotech., CA, USA). After two 15 min washes in PBS, cells were incubated in 1:100 FITC-conjugated goat anti-mouse IgG or Texas Red-conjugated goat anti-rabbit IgG. Both secondary antibodies were from Vector Laboratories (Burlingame, CA, USA). Nuclei were counterstained with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI; Roche, Mannheim, Germany) for 2 min.

After extensive washing, the samples were mounted with PBS/glycerol (1:1) and covered with a coverslip. Images were immediately acquired with a Nikon Eclipse E600 microscope (Nikon, Italy) equipped with epifluorescence optics, connected to a Nikon digital camera DXM1200, and processed with Nikon AC-1 software.

Confocal laser microscopy and image processing analysis. Co-localization was performed by confocal laser fluorescence microscopy. Confocal imaging was performed on Olympus fluoview FV1000 confocal microscope equipped with FV10-ASW version 1.6 software, multi-line argon (T=458-488 and 514 nm) and 2X helium/neon (T=543 and 633 nm) lasers

Table I. Effect of different concentrations of H_2O_2 on growth of HepG2 and 3B cells. Data are presented as number of cells x10^5.

	NT[a]	50 μM	100 μM	150 μM	200 μM	500 μM
HepG2						
0 h	3.30±0.19	3.31±0.22	3.33±0.28	3.29±0.26	3.30±0.16	3.25±0.20
48 h	12.62±0.91	11.44±0.87	10.65±0.79[c]	7.20±0.55[b]	6.98±0.59[b]	5.03±0.36[b]
Hep3B						
0 h	3.10±0.20	3.08±0.17	3.10±0.23	3.04±0.19	3.07±0.26	2.98±0.19
48 h	14.11±1.13	12.28±1.09[d]	10.9±0.94[c]	7.02±0.62[b]	6.74±0.46[b]	5.10±0.38[b]

[a]NT, non-treated cells. Values are means (±SEM) of three independent experiments repeated in triplicate. [b]P<0.001; [c]P<0.01; [d]P<0.05 versus NT value.

Table II. Effect of different concentrations of NAC on growth of HepG2 and 3B cells. Data are presented as number of cells x10^5.

	NT[a]	1 mM	2 mM	4 mM	5 mM	6 mM
HepG2						
0 h	3.27±0.29	3.33±0.25	3.38±0.23	3.39±0.28	3.38±0.19	3.40±0.22
48 h	12.48±0.86	13.16±1.08	17.91±1.25[b]	18.18±1.30[b]	18.83±1.43[b]	20.09±1.69[b]
Hep3B						
0 h	3.02±0.15	3.11±0.27	3.12±0.21	3.10±0.21	3.15±0.20	3.08±0.18
48 h	12.88±1.10	14.07±1.15[c]	18.17±1.63[b]	18.95±1.58[b]	19.12±1.57[b]	19.90±1.61[b]

[a]NT, non-treated cells. Values are means (±SEM) of three independent experiments repeated in triplicate. [b]P<0.001; [c]P<0.05 versus NT value.

with 60X, 1.42 N.A. oil immersion objective. Co-localization analysis for dual stained samples was carried out using the FV10-ASW co-localization function, and images were finally processed with Adobe Photoshop 9.0.

Western blotting. At the indicated time points, cell extracts were prepared in ice-cold RIPA lysis buffer containing, 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1% sodium deoxycholate, and 10% cocktail protease inhibitors (Roche). Equivalent amounts of protein were re-suspended in Laemmli sample buffer (26) and resolved on SDS-polyacrylamide gel electrophoresis. The proteins were then transferred and immobilized on PVDF membranes (Amersham, Germany), blocked with 5% nonfat dried milk and probed with appropriate primary and secondary antibodies. Immunoblots were visualized with the ECL detection system (Amersham). All antibodies were purchased from Santa Cruz.

NF-κB glutathionylation. Protein of cellular extracts (100 μg) were incubated overnight at 4˚C with 1 μg of anti-GSH antibody, and 15 μl of the resuspended volume of protein A/G PLUS-agarose (Santa Cruz) were added to the solution and incubated at 4˚C for 1 h on a rocker platform. The pellet was collected by centrifugation at 800 x g for 30 sec at 4˚C and washed four times with PBS. After a final wash, the pellet was resuspended in 10 μl of Laemmli sample buffer, boiled for 2 min, loaded onto an SDS-polyacrylamide gel for Western blot analysis, and revealed with anti-p65 antibody (1:500).

Statistical analysis. Data are presented as mean ±SEM for at least 4 independent experiments and evaluated using Student's t-test. Values of $p<0.05$ were considered significant.

Results

Proliferative and apoptotic response was dose-related in hepatoma cells treated with H_2O_2 or NAC. It is well known that in different liver cells H_2O_2 exerts a genotoxic action whereas pharmacological actions of NAC include antioxidant and -inflammatory effects (27). However, poor data demonstrate the ability of H_2O_2 and NAC to exert their pro- and anti-oxidant action in hepatoma cell lines. Furthermore, the window within which H_2O_2 and NAC are capable to promote/inhibit both apoptosis and cell proliferation in this *in vitro* model of hepatic cells is still unknown (28-30).

Thus, here we exposed two hepatoma cell lines (HepG2 and 3B) to growing amounts of H_2O_2 or NAC, from physiological to nonphysiological concentrations. The chosen amounts were, 50, 100, 150, 200 and 500 μM for H_2O_2, and 1, 2, 4, 5 and 6 mM for NAC. After 30 min H_2O_2-treatment and 1 h NAC-treatment, culture mediums were replaced and cells were harvested and collected at different time points (0, 4, 24

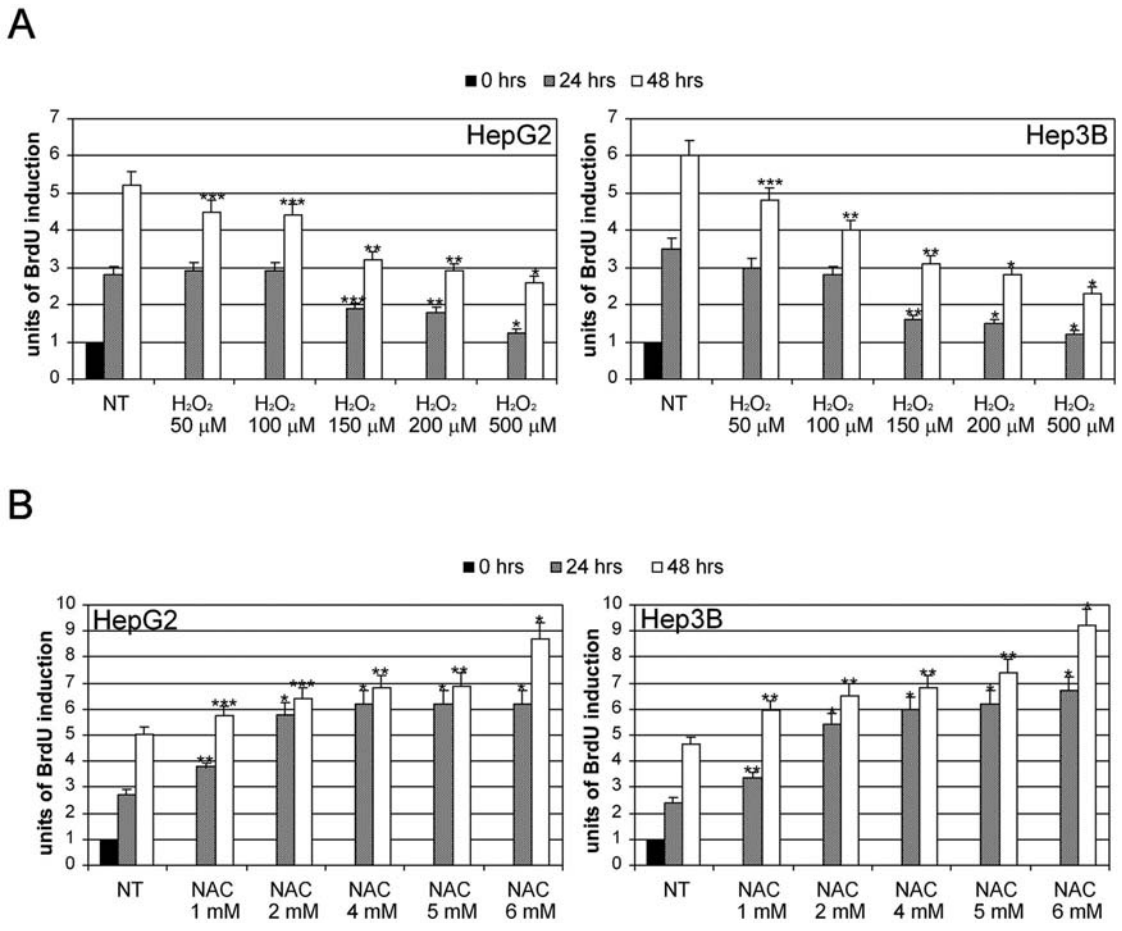

Figure 1. Effect of H_2O_2 and NAC on DNA synthesis of HepG2 and 3B cells. Confluent HepG2 and 3B cells (30-40%) were exposed or not (non-treated, NT) to different concentrations of H_2O_2 (A) or NAC (B). H_2O_2 was maintained in medium for 30 min and NAC for 1 h. After treatment culture medium was replaced and analysis of DNA synthesis was performed at three different time points, 0, 24 and 48 h after treatment. DNA synthesis was evaluated as incorporation of BrdU. Quantitative data of the analysis of BrdU incorporation was converted to unit of induction with respect to NT sample considered as 1. Histograms are the mean value (±SEM, bars) of five independent experiments. $^{*}P<0.001$; $^{**}P<0.01$; $^{***}P<0.05$ versus NT value.

and 48 h) to examine, by a dose-response curve, the rate of cell growth, DNA synthesis and apoptosis. Data obtained from treated cells were compared with untreated control cells (see Materials and methods for details).

The rates of cell growth after treatments was evaluated by Trypan-blue staining and are reported in Tables I and II. Analysis of results showed primarily that the behaviour in relation to cell growth was the same for both HepG2 and 3B cell lines. In particular, after 48 h with growing doses of H_2O_2, cell growth progressively decreased with respect to control cells (Table I), whereas exposure of cells with growing doses of NAC caused an increase in cell growth which reached a plateau at a value exceeding 2 mM (Table II).

Secondly, we evaluated the rate of cell proliferation by a colorimetric assay for BrdU incorporation which provides a quantitative analysis of DNA synthesis (Fig. 1A and B). No relevant differences were found between the two hepatoma cells. As shown in Fig. 1A, during the first 24-48 h after treatment with H_2O_2, we observed a progressive dose-dependent decrease of DNA synthesis in treated cells with respect to control cells. Interestingly, DNA synthesis reached a plateau after 2 mM NAC, in agreement with Trypan blue measurements shown earlier in Table II (Fig. 1B). Finally, we performed the analysis of apoptotic early (4 h) and late (24 h) rates using an ELISA test which was able to evaluate the nucleosome fragments (DNA-fragmentation) (Fig. 2A and B). Significant variations of apoptosis were observed only after 24-h. In particular our data indicate that H_2O_2 did not affect apoptotic rate at doses <150 μM, whereas it induced a significant increase of apoptosis at concentrations ≥200 μM (Fig. 2A). Surprisingly, as shown in Fig. 2B, NAC was also able to induce apoptosis augmentation after treatments exceeding the 4 mM value. The apoptotic response in HepG2 cells was similar to Hep3B.

Taken together, these observations suggest that the treatment with an anti-oxidant agent, such as NAC, at high doses impairs, as well as occurs with H_2O_2, the balance between proliferation and apoptosis in hepatoma cells.

H_2O_2 and NAC influences the amount of free and protein-bound glutathione. Different forms of glutathione were observed in biological samples, reduced glutathione fraction (GSH), oxidized glutathione disulfide (GSSG), and oxidized disulfide form with various thiol-containing proteins (ProSSG) (5,31).

Results reported above demonstrated that the proliferative and apoptotic effects of H_2O_2 and NAC were strictly dose-dependent, thus we chose to examine whether the minimal and

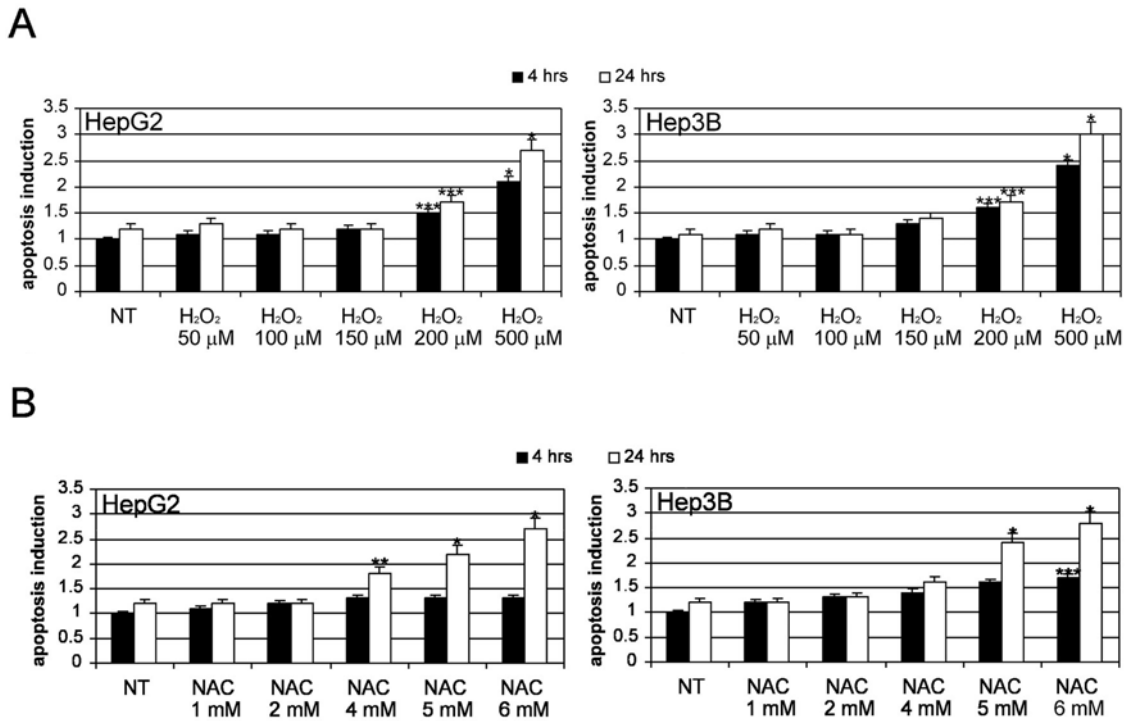

Figure 2. Effect of H_2O_2 and NAC on apoptosis of HepG2 and p3B cells. Confluent HepG2 and 3B cells (30-40%) were exposed or not (non-treated, NT) to different amounts of H_2O_2 (A) or NAC (B). H_2O_2 was maintained in medium for 30 min and NAC for 1 h. After treatment, culture medium was replaced and analysis of apoptosis was performed at three different time points, 0, 4 and 24 h after treatment. Apoptosis was evaluated by quantification of mono- and oligonucleosomes. The number of apoptotic cells was transformed in unit of induction with respect to non-treated sample considered as 1. Histograms are the mean value (±SEM, bars) of four independent experiments. $^{*}P<0.001$; $^{**}P<0.01$; $^{***}P<0.05$ versus NT value.

maximal effective doses of H_2O_2 (50 and 500 μM) and NAC (1 and 6 mM) were able to influence, immediately after treatment (time point 0 h), the amounts of different forms of glutathione. Since identical results were obtained with the two examined cell lines, only results from Hep3B cells are presented. The amount of different forms of glutathione were analyzed by HPLC, thus GSSG/GSH and ProSSG/GSH Tot ratios are shown (Fig. 3). ProSSG/GSH Tot ratio and GSSG/GSH ratio were significantly increased in H_2O_2-treated hepatoma cells with respect to control cells, while 6 mM NAC significantly reduced ProSSG/GSH Tot ratio compared to controls.

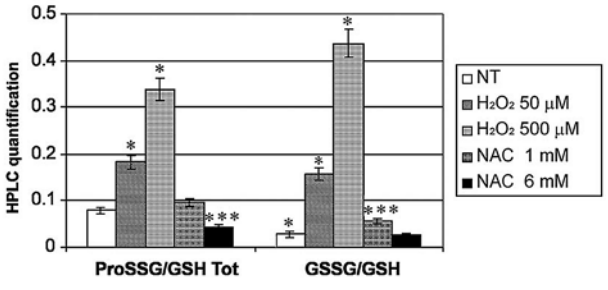

Figure 3. Effect of H_2O_2 and NAC on different GSH forms in Hep3B cells. Confluent cells (30-40%) were exposed or not (non-treated, NT) to 30 min treatment with H_2O_2 (50 or 500 μM) or 1 h treatment with NAC (1 or 6 mM). After incubation HPLC analysis of different GSH forms was conducted. The GSSG/GSH and ProSSG/GSH Tot ratios were determined in hepatoma cells. For details, see Materials and methods. Histograms are the mean value (±SEM, bars) of two independent experiments. SEM<10%.

These results are also confirmed by the analysis of protein glutathionylation in both hepatoma cells. We performed an immunofluorescent analysis by a double staining with an antibody recognizing all glutathionylated proteins and DAPI in cells at time point 0. Since the pattern of glutathionylated proteins were the same in both hepatoma cells, we show only the panel resuming immunofluorescence results obtained in Hep3B cells (Fig. 4). As indicated in Fig. 4A-J, whatever the dose used, the treatment with H_2O_2 caused an increased protein glutathionylation, whereas the treatment with NAC was ineffective on immunofluorescent staining of glutathionylated proteins. Importantly, glutathionylated proteins were equally distributed in the cytoplasm and cytoskeleton but completely absent in the nucleus.

H_2O_2 and NAC affected glutathionylation and nuclear translocation of NF-κB. We tested the possibility that one of the glutathionylated proteins is NF-κB. We performed an immunoprecipitation assay after 2-h treatment with H_2O_2 (50 and 500 μM) and NAC (1 and 6 mM). We immunoprecipitated H_2O_2- and NAC-treated cell extracts with an antibody recognizing glutathione and all glutathionylated proteins, which in turn were analyzed by Western blotting for the p65 subunit of NF-κB. As shown in Fig. 5, p65 is glutathionylated in both hepatoma cells. Interestingly, the treatment with NAC induces a decrease of a glutathionylated form of p65 in our hepatoma cells, whereas no significant difference was observed after treatment with H_2O_2.

Variations of p65 glutathionylation might influence its nuclear translocation, which is a prerequisite for NF-κB

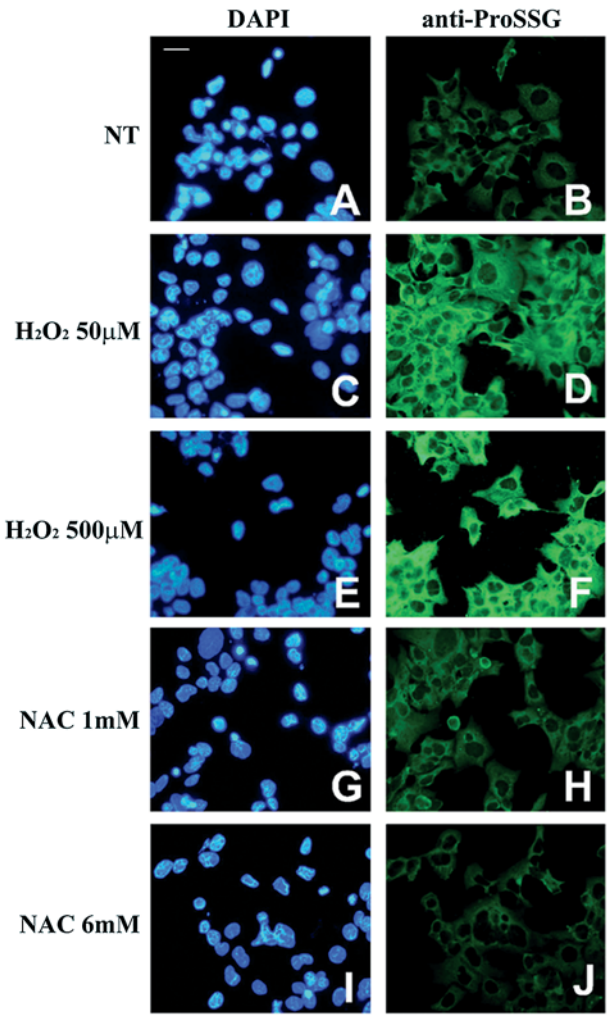

Figure 4. Immunofluorescence of glutathionylated proteins after treatment with different doses of H_2O_2 (50 or 500 μM) and NAC (1 or 6 mM), compared whith non-treated cells (NT). Samples were prepared for immunocytochemistry using anti-glutathionylated protein antibody (anti-ProSSG) and a FITC-conjugated anti-mouse IgG (B, D, F, H, J, green). DAPI (A, C, E, G, I, blue) was included to stain nuclei. Fluorescence was acquired with a Nikon Eclipse E600 microscope, connected to Nikon digital camera DXM1200. All images were processed with Nikon AC-1 software. Magnification bar, 30 μm.

activity as a transcriptional factor. Thus, we analysed the intracellular localization of p65 and intracellular distribution of glutathionylated proteins by laser scanning confocal immunofluorescence microscopy. As shown in Fig. 6, red immunofluorescence revealed that the treatment of cells with H_2O_2 maintains p65 preferentially in the cytoplasmic and perinuclear region (Fig. 6B and C). Conversely, the translocation of p65 into the nucleus was observed following treatment with NAC, overall at a 1-mM dosage (Fig. 6D). Green immunofluorescence confirmed data already demonstrated by Fig. 4, a widespread distribution of glutathione-protein complexes, excluding the nucleus, and a greater amount of protein-bound glutathione after exposure to H_2O_2. Interestingly, the combination of red and green staining, revealed that p65 co-localized with protein-bound glutathione only at the cytoplasmic and perinuclear level (Fig. 6Q and R), while p65 was completely untied by glutathione when localized into the nucleus.

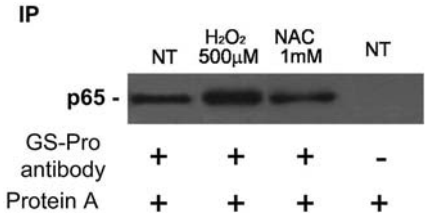

Figure 5. p65NF-κB glutathionylation in HepG2 cells. HepG2 H_2O_2- (500 μM) and NAC-treated (1 mM) cells were analysed immediately after treatment (time point 0), lysed and subjected to immunoprecipitation with anti-glutathione-protein complexes antibody. Immunocomplexes (lanes 1-3) and total lysates (lane 4) were incubated for 1 h with protein A/G agarose. Western blotting was performed on pellets to reveal p65 subunits of NF-κB. A representative gel from one of three separate experiments is shown.

Discussion

We found that, depending on the dose, both H_2O_2 and NAC induce different cellular responses in two different hepatoma cell lines, in agreement with others (32,33). H_2O_2 was able to stop proliferative response proportionally to its concentration, whereas it induced apoptosis only when used at a high dosage (500 μM) (Figs. 1 and 2). On the contrary, NAC increased DNA synthesis and a significant increase of apoptosis was observable only at very high doses (6 mM) (Figs. 1 and 2). It is not surprising, that although NAC acts as a glutathione precursor and direct anti-oxidant, some studies showed that excessive amounts of NAC act as pro-oxidants inducing oxidative stress, redox status imbalance and excessive apoptosis (34). On the other hand, the proproliferative ability of NAC is not yet documented in hepatocytes. However, this biological phenomenon is only contradictory for liver cell physiology. In fact, the regenerative ability of the liver is strictly linked to the balance between proliferation and apoptosis, and several agents (i.e. retinoic acid) at specific concentrations act as cytostatic agents to maintain this equilibrium (35,36). In a similar scenario, high doses of pro-oxidants dangerously raise the apoptotic rate, but also an excessive dosage of anti-oxidant agents (such as NAC and glutathione), may be equally damaging.

Oxidative stress may induce hepatocyte damage and death by altering bio-molecules, including DNA, proteins, and lipids (37). Oxidative stress-induced modification of proteins occurs in several ways. However, the most important modification is glutathionylation (38). Some interesting studies suggest that protein glutathionylation is a physiologically relevant mechanism for controlling magnitude of activation of NF-κB (39,40). However, no evidence demonstrates the existence of a possible role of NF-κB glutathionylation in hepatocyte damage.

The main finding of the present study is that different doses of H_2O_2 and NAC alter the proliferation/apoptosis balance in hepatoma cells. Moreover, proliferative response correlates well with a major nuclear translocation of p65NF-κB, while the apoptotic picture associates with an increased p65NF-κB glutathionylation. In apoptotic cells, p65NF-κB localized principally into the cytoplasm and was majorly glutathionylated, whereas proliferating cells displayed a predominantly nuclear localization of glutathione-free p65 (Fig. 6). These results suggest that glutathionylation has a

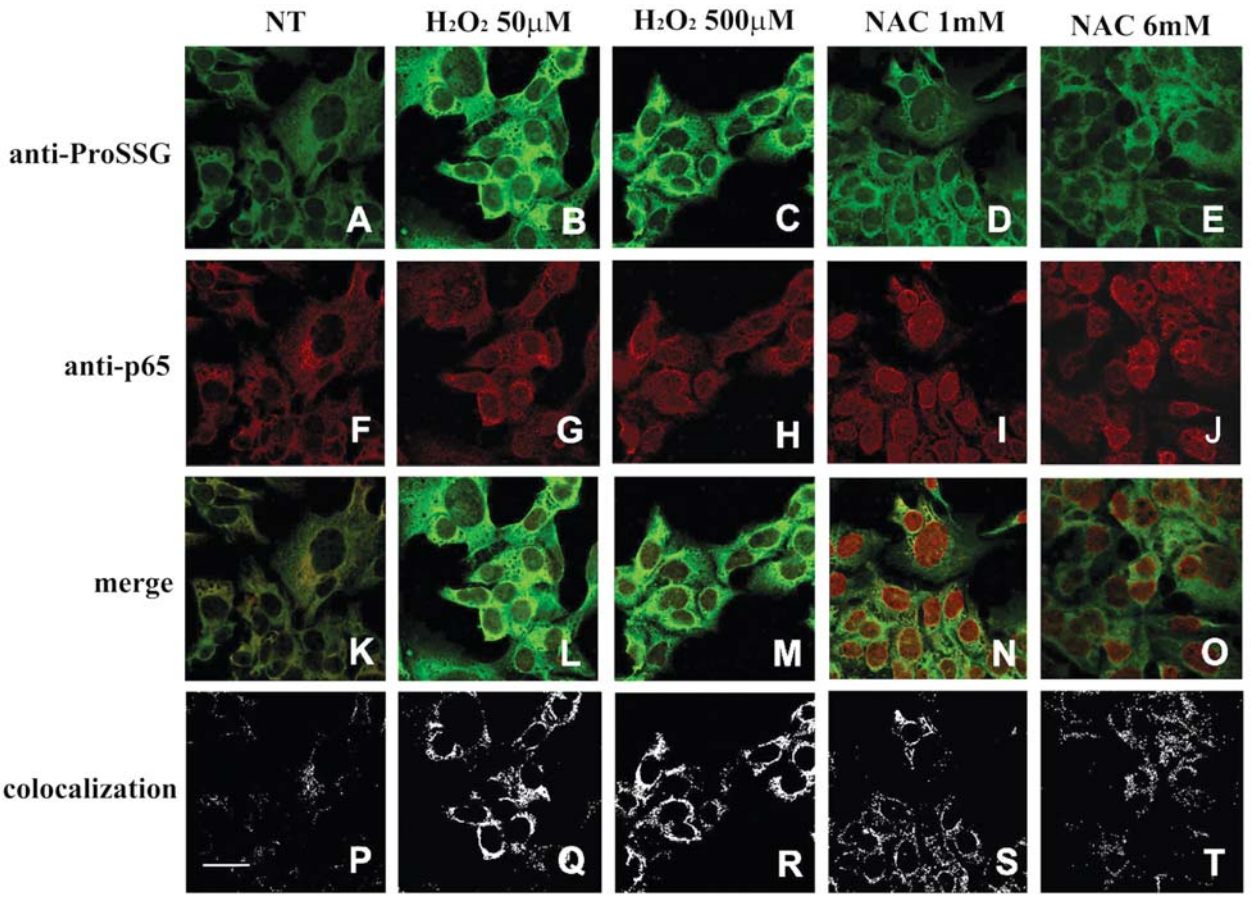

Figure 6. Effect of H_2O_2 and NAC on p65NF-κB nuclear translocation. Hepatoma cells were untreated or treated as already described. The nuclear translocation of p65 was assessed by confocal fluorescence microscopy using anti-p65 antibody (F-J, red). Gluathione-protein complexes were also revealed (A-E, green). Merge images (green-red) are showed from K to O, whereas co-localization are represented from P to T. Only co-localization points of p65NF-κB with protein-binding glutathione are reported in white. Magnification bar, 30 μm.

role in the transcriptional activity of p65NF-κB, but further investigations are required in this field.

Deletion of p65 is embryonically lethal owing to increased hepatocyte apoptosis, thus it has a central role in liver pathophysiology (41). Particularly, because it is critical for maintaining balance between hepatocyte proliferation and apoptosis, NF-κB exerts a major influence on a large number of acute and chronic liver diseases (42). Increased apoptosis in the liver may contribute to tissue inflammation, fibrogenesis, and development of cirrhosis (43). Interestingly, apoptosis, degrees of inflammation and liver fibrosis correlated with the p65NF-κB expression (44). Furthermore, a major function of NF-κB in liver myofibroblasts (hepatic stellate cells) is the inhibition of apoptosis (45,46). The NF-κB pathway is also one of the most important signalling pathways during development and progression of alcoholic and non-alcoholic steatohepatitis (ASH/NASH) (47,48). All these liver diseases, especially NASH, which is a more advanced form of NAFLD, are characterized by pathogenetic mechanisms including, oxidative stress and redox state imbalance (49-51).

In conclusion, our results suggest, for the first time, that glutathionylation of p65 subunit of NF-κB is a critical factor leading to apoptosis and consequently to the alteration of redox status of hepatocytes. Moreover, since we already demonstrated an increase of protein glutathionylation in the liver of paediatric patients with NAFLD (25), further studies are needed to understand the role of glutathionylated-p65NF-κB in the pathogenesis of this liver disease. We also noted that although antioxidants, such as NAC, interfere with NF-κB activity at different levels and may be useful for the treatment of liver diseases, we must be very careful of the optimal doses administered to patients, because p65NF-κB also plays an important hepatoprotective function that needs to be taken into consideration during development of new therapeutic regimens (52).

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