Paradoxical effect of diphenyleneiodonium in inducing DNA damage and apoptosis

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Abstract:

Diphenyleneiodonium (DPI) is often used as a molecular tool in unravelling redox-sensitive cellular events involving NADPH oxidase. However, to better understand unexpected actions of DPI, it was ascertained if DPI affects cellular DNA. DPI induced single-strand breaks in DNA of HCT-116 cells, although this only slightly increased GADD153 expression. Nevertheless, after sustaining DNA damage, the DPI-treated cells subsequently had features characteristic of apoptosis, such as translocated membrane phospholipid and nuclei containing condensed chromatin. Paradoxically, DPI attenuated the DNA damage and overall ROS production caused by sodium deoxycholate (DOC), although DPI did not inhibit DOCinduced generation of mitochondrial O_2–. Furthermore, DPI prevented the occurrence of apoptosis caused by DOC. However, other known chemical inhibitors of NADPH oxidase did not produce the same results as DPI in negating the effects of DOC. Collectively, these disparate findings suggest that DPI can act not in accord with conventional wisdom depending on the experimental conditions.

Keywords:

Apoptosis, deoxycholate, diphenyleneiodonium, DNA damage, NADPH oxidase

Article:

INTRODUCTION

Reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2) [1], have the capacity to initiate signalling cascades leading to gene activation. One primary source of H_2O_2 originates from the superoxide anion (O_2•–) produced from the reaction catalysed by the plasma membrane-localized flavoenzyme known as NADPH oxidase [2]. Accordingly, NADPH oxidase is involved in mediating signal transduction and redox-sensitive gene expression. This has attracted considerable interest because links have been made between NADPH oxidase and the development of various chronic diseases associated with oxidative stress and inflammation [3-5].

To investigate any involvement of NADPH oxidase in mediating signal transduction and redox-sensitive gene expression, it is a common experimental approach to utilize various chemical inhibitors of NADPH oxidase. Despite being a broad-spectrum flavoenzyme inhibitor, diphenyleneiodonium (DPI) is often perceived as one of the most popular NADPH oxidase inhibitors. Conceptually, if DPI impedes or prevents the molecular event being investigated, then the basic conclusion would generally be drawn that NADPH oxidase plays a role in the event. However, this may not always be valid. Although DPI can inhibit the production of O_2•– by NADPH oxidase, it is also known to inhibit the production of O_2•– and also H_2O_2 by mitochondria in monocytes/macrophages [6].

On the other hand, DPI can have other cellular effects besides inhibiting NADPH oxidase. One effect in particular is contradictory in that DPI has been reported to actually induce the production of O_2•– [7], although

this finding is not in agreement with another study [8]. Nevertheless, it has been demonstrated that DPI can induce oxidative stress, based on results showing that DPI increases the production of H_2O_2 in N11 mouse glial cells [9]. Evidence of lipid peroxidation was found. Moreover, DPI changed the cellular redox status by causing a decrease in glutathione and an increase in glutathione disulphide. Therefore, depending probably on the experimental conditions, it would appear that DPI is capable of either reducing or promoting oxidative stress in cells, considering DPI's conflicting ability to both inhibit and stimulate the formation of O2•– or downstream products. In the case of the latter situation, it is not known if DPI causes structural damage to susceptible cellular components such as DNA.

The ability of DPI to induce ROS formation [9] is an effect produced also by bile acids [10]. Bile acids may promote colonic abnormalities associated with inflammation [11], which is frequently associated with ROS. Previously, we reported that the bile acid (or salt), sodium deoxycholate (DOC), induced DNA damage and apoptosis in HCT-116 human colon epithelial cells [12]. Hence, ROS may have been involved in causing the effects of DOC. This probability is supported by other studies [13,14], where certain antioxidants were shown to decrease the number of rat hepatocytes undergoing apoptosis upon exposing these cells to glycochenodeoxycholic acid. Based on the finding that bile acids induce the formation of ROS [10], along with our suspicion that NADPH oxidase might be involved, we were prompted to investigate whether DPI could negate the effects of DOC. In doing so, we now report a paradoxical effect of DPI, specifically its ability to cause cellular DNA damage and apoptosis, but actually prevent the cellular DNA damage and apoptosis caused by DOC.

MATERIALS AND METHODS

Materials

HCT-116 human colon adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). DOC, DPI and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Cell culture and treatment

HCT-116 colonocytes were propagated in McCoy's 5A medium that was supplemented with 100 ml/L foetal bovine serum, 2 mmol/L glutamine, 0.54 µmol/ L fungizone, 100 000 units/L penicillin and 100 mg/L streptomycin.

Upon reaching 70-80% confluency, the cells were exposed to 0-50 µM DPI for 0-40 h depending on the experiment. In other experiments, cells were pretreated with 10 µM DPI, 100 µM apocynin, 10 µM neopterin or 1 mM Tiron for 0.5 h before exposing them to 600 µM DOC for 3 h. These concentrations are consistent with what numerous workers in the field use.

Comet assay

Cellular DNA damage was assessed by performing alkaline gel electrophoresis with visualization by fluorescence microscopy (comet assay), as described previously [12]. For each sample, each of 50 randomly selected nucleoids was scored on a numerical scale of 0-4 to arrive at the total comet score. Data are expressed as the average ± SEM.

Assessment of overall ROS production, mitochondrial superoxide anion generation and mitochondrial membrane potential (MMP) status

Utilizing fluorescence microscopy, overall cellular ROS production was assessed with an Image-iT LIVE Green Reactive Oxygen Species Detection Kit (Molecular Probes, Inc., Eugene, OR, USA). Mitochondrial superoxide anion generation and MMP status were assessed with the MitoSOX Red reagent (Molecular Probes, Inc., Eugene, OR, USA) and DePsipher reagent (Trevigen, Gaithersburg, MD, USA), respectively.

Determination of GADD153 expression

The expression levels of GADD153 mRNA and protein were determined by multiplex relative RTPCR analysis and Western-immunoblotting analysis, respectively, as described previously [15]. For mRNA expression, relative GADD153 mRNA (GADD 153: β-actin ratio) was computed from analysis of stained gels on a Kodak Image Station 440 CF imaging system.

Annexin V-Alexa Fluor 488 binding assay

HCT-116 cells that had been grown and treated with the test agents in chamber slides were washed twice with cold phosphate-buffered saline. The washed cells were equilibrated for 10 min in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4). After equilibration, the binding buffer was removed from the chamber slide. Then, fresh binding buffer but containing annexin V-Alexa Fluor 488 conjugate (Molecular Probes, Eugene, OR) was pipetted into the chamber slide for 15 min of incubation in the dark (CO_2 incubator). After washing with binding buffer and counter-staining with propidium iodide, cells on the slides were examined by fluorescence microscopy.

Determination of chromatin condensation/fragmentation

After washing and fixation of the cells, they were stained with 4',6-diamidine-2-phenylindole (DAPI) and then examined by fluorescence microscopy, as detailed previously [12]. In some experiments, the percentage of cells having chromatin condensation/ fragmentation was calculated after examining 100 randomly selected cells over the entire surface of the slide. Data are expressed as the average ± SEM.

RESULTS

Effect of DPI on DNA structural integrity

DPI is known to induce the formation of H_2O_2 in cells resulting in the peroxidation of lipids [9] . To determine if DPI is capable of also causing damage to DNA, HCT-116 cells were exposed to DPI for subsequent analysis by the comet assay to detect any single-strand breaks in DNA. As can be seen by the first set of images (Figure 1A) and the corresponding bar graph (Figure 1B), DPI caused cellular DNA damage in a concentration-dependent manner within the range of 0-20 µM. Untreated control cells had nucleoids that were spherical, reflecting no DNA damage and producing a comet score of 0 ± 0. In contrast, DPI-treated cells had nucleoids with the typical comet appearance, reflecting DNA single-strand breaks. The comet tails became more prominent, which is indicated also by the increasing comet scores, as the cellular DNA damage became more extensive with increasing DPI concentrations. Additionally, opting to use a higher concentration of DPI (50 µM) in order to best see how soon DPI causes comets to appear, it was determined that the DNA-damaging effect of DPI was essentially time-dependent beginning as early as 0.5 h (Figure 1C). To determine if antioxidants could inhibit the effect of DPI (Figure 1D), two substances were tested. Tiron (4,5-dihydroxy-1,3-benzene disulphonic acid), which purportedly scavenges O2•– [15], inhibited DPI- induced DNA damage. In contrast, N-acetylcysteine (NAC), which increases intracellular glutathione [16], did not produce such an inhibitory effect.

Effects of DPI, DOC and Tiron on production of ROS and functioning of mitochondria

Based on an assay that detects the oxidation by ROS of the reduced form of 5- (and 6-)carboxy-2',7'-dichlorodihydrofluorescein diacetate (non-fluorescent) to a product having fluorescence, DOC markedly increased overall production of ROS in the cells (Figure 2A). The effect of DOC was inhibited by either DPI or Tiron, with the former being more effective. This particular assay did not provide visual evidence that DPI increases ROS production (Figure 2A). However, based on a more specific detection assay using MitoSOX Red reagent, it would appear that DPI slightly increased mitochondrial superoxide anion generation, whereas DOC had a more noticeable effect (Figure 2B). Interestingly, as further shown in the image set, DPI did not inhibit the potentiation of mitochondrial superoxide anion generation caused by DOC. In assessing mitochondrial functioning, i.e. MMP status, with the DePsipher reagent (Figure 2C), DPI, and DOC to a lesser extent, caused collapse or loss of MMP as indicated by the reduction in fluorescence intensities of the DPI-treated and DOC-treated cells in comparison to control cells.

Effect of DPI and DOC on GADD153 expression

It is known that DNA damage can result in upregulation of the growth arrest and DNA damage-inducible genes (GADD), particularly GADD153. For example, we previously reported that DOC damages cellular DNA [12], resulting in upregulation of GADD153 mRNA and protein expression [17]. Thus, because DPI caused DNA damage as well, we asked the question whether the expression of GADD153 mRNA and protein is changed in HCT116 cells exposed to 50 μM DPI, a concentration that we would expect to more likely find any potential effect on GADD 153. Although DPI slightly increased GADD153 mRNA (Figure 3A), GADD153 protein expression was not induced by DPI (Figure 3B), in contrast to the effect of DOC. In other experiments, DPI did not prevent upregulation of GADD153 mRNA caused by DOC (Figure 3C). In contrast, the O2•– scavenger, Tiron, prevented DOC-induced upregulation of GADD153 mRNA (Figure 3D). Our previous study [17] reported that NAC, α-tocopherol and catalase were ineffective.

DPI attenuates DOC-induced DNA damage

As shown by the images (Figure 4A) and scoring results (Figure 4B), DOC caused noticeable DNA damage in HCT-116 cells, producing a comet score of 72 ± 30. Likewise, DPI also caused DNA damage, although not as extensive (comet score of 44 ± 15). Most notable, however, pre-treatment of the cells with DPI reduced the extent of the DNA damage caused by DOC, as reflected by a lower comet score of 42 ± 17. Thus, while DPI by itself caused DNA damage, DPI was able to lessen the DNA damage caused by DOC.

DPI causes apoptosis but inhibits the onset of apoptosis caused by DOC

In view of the significant DNA damage in HCT-116 cells exposed to DPI, the initiation of apoptosis would be a predictable consequence. During apoptosis,

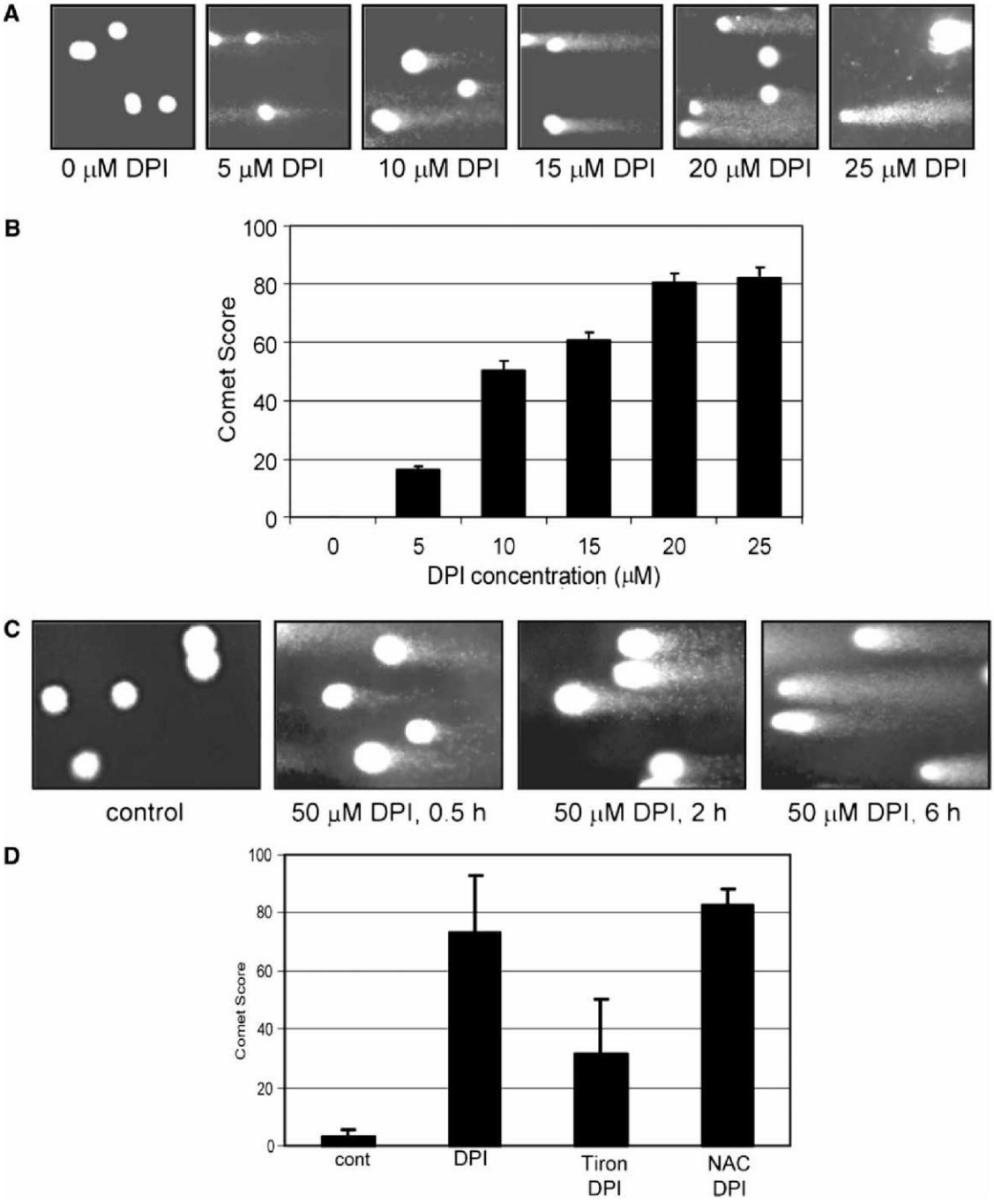

Figure 1. DPI induces DNA damage and effect of antioxidants. HCT-116 cells were incubated with 0–25 μM DPI for 3 h. DNA damage was assessed by the comet assay, in which images (representative of three different experiments) were captured (A) and corresponding comet scores (average ± SEM, $n = 3$) were attained (B). Additionally, cells were incubated with 50 μM DPI for 0–6 h and the comet assay was performed to generate the images representative of three different experiments (C). In the last experiment (D), cells were pre-treated with 20 mM of either Tiron or NAC for 2 h. Then, they were exposed to 25 μM DPI for 3 h, after which comet scores (average ± SEM, $n = 3$) were attained.

phosphatidylserine is known to translocate from the inner to outer leaflet of the plasma membrane. To determine if such an event happens in DPI-treated HCT-116 cells, the annexin V-Alexa Fluor 488 binding assay was performed after 20 h of incubation of the cells with a broad concentration range of DPI in order to see any potential dose-response relationships (Figure 5A). As can be seen, in a concentration-dependent manner, more of the DPI-treated cells in each case (10, 30, 50 μM) than the untreated control cells (0 μM) bound the annexin V-Alexa Fluor 488 probe (green fluorescence) at the cell surface. Incidentally, propidium iodide (red fluorescence) entered some of the DPI-treated cells, indicating that the plasma membrane of these cells had been breeched as apoptosis reached the advanced stages.

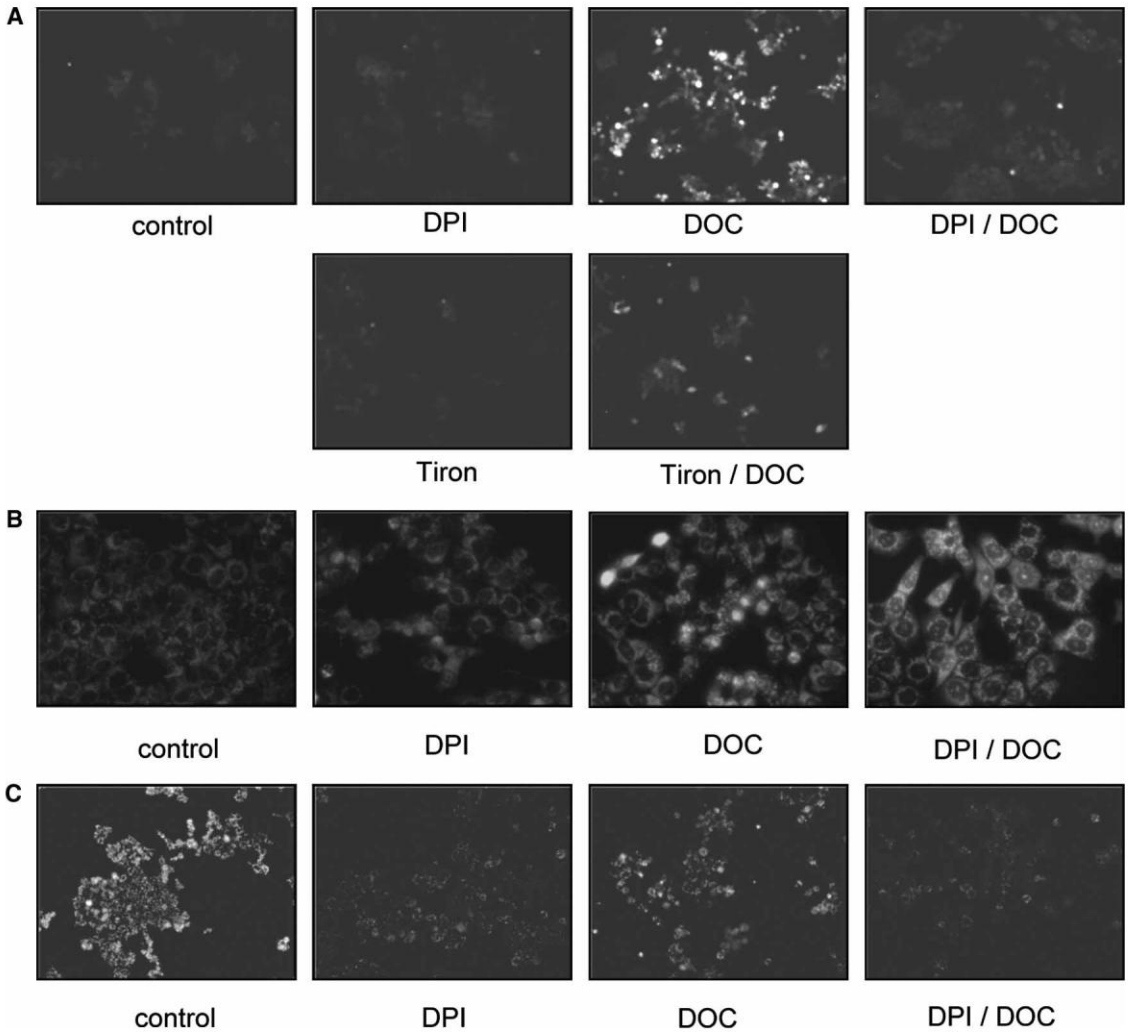

Figure 2. Effects of DPI, DOC and Tiron on ROS production and mitochondrial functioning. HCT-116 cells were pre-treated with 10 μM DPI or 20 mM Tiron for 0.5 h and exposed to 300 μM DOC for 2 h. Afterwards, cells were stained to assess cellular ROS production overall (A), mitochondrial superoxide anion generation (B) and mitotochondrial membrane potential status (C). All of the fluorescence-based images are representative of at least three different experiments.

In other experiments, when cells were examined at 6 h (Figure 5B), relatively few of the DPI-treated cells had condensed chromatin in nuclei, which is another feature characteristic of apoptosis. However, at 40 h, chromatin condensation was present in many of the DPI-treated cells (Figure 5C). However, substantial fragmentation of chromatin was not observed.

We next asked the question if 10 μM DPI is able to influence the capacity of 600 μM DOC to induce apoptosis. Morphological features characteristic of apoptosis were clearly evident in DOC-treated HCT116 cells, including membrane blebbing and breakup of the cells into apoptotic bodies (Figure 6A). Furthermore, DOC-treated cells had greater binding of annexin V-Alexa Fluor 488 (Figure 6B). Also, DOC caused chromatin condensation/fragmentation, as can be seen in the images (Figure 6C) and corresponding bar graph (Figure 6D). More specifically, chromatin condensation/fragmentation was present in 299 17% of the DOC-treated cells examined. However, when pre-treated with DPI and then exposed to DOC, none of the cells examined had any chromatin condensation/fragmentation. At this relatively low concentration of DPI tested, it is worth noting that the cells seem to be still undergoing mitosis, based on the observance of separating sister chromatids like the ones seen in control cells (Figure 6C, and also Figure 5B). As can be further seen, membrane blebbing and apoptotic bodies, as well as annexin V-Alexa Fluor 488 binding, were not found appreciably in HCT-116 cell samples that were pre-treated with DPI for 0.5 h and then exposed to DOC. Similar non-apoptotic results were obtained when cells were co-treated with DPI and DOC at the same time, but morphological features of

apoptosis were observed in cells treated with DPI 0.5 h, 1 h and 2 h after being initially dosed with DOC (data not shown). Interestingly, in contrast to DPI, two other

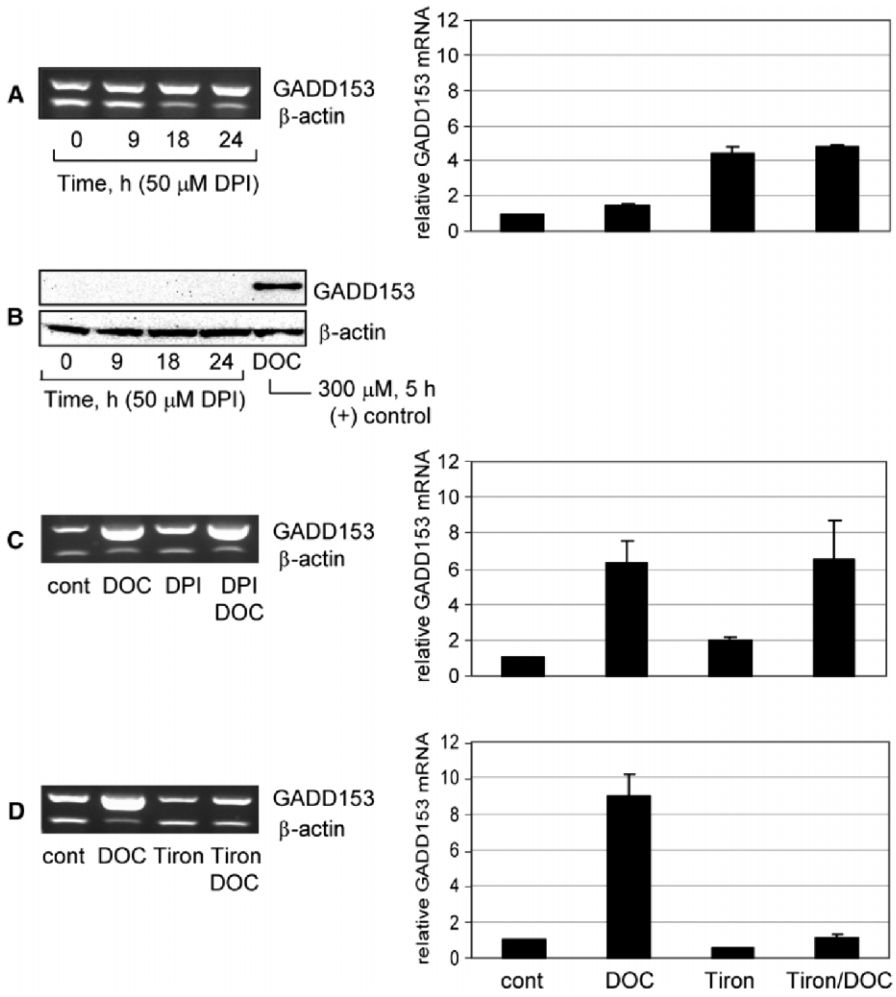

Figure 3. Effects of DPI, DOC and Tiron on GADD153 expression. HCT-116 cells were incubated with 50 μM DPI for 0–24 h. Then, multiplex relative RT-PCR analysis (A) was performed to determine mRNA expression of the target gene, GADD153, and internal control gene, *β*-actin. In conjunction, Western-immunoblotting analysis (B) was performed to determine GADD153 protein expression (*β*-actin—loading control), with DOC-treated cells serving as a positive control. The results from three different experiments were similar. In the last two experiments, cells were pre-treated with 10 μM DPI (C) or 20 mM Tiron (D) for 0.5 h and exposed to 300 μM DOC for 5 h, before GADD153 mRNA analysis.

commonly used NADPH oxidase inhibitors (neopterin and apocynin) did not have any inhibitory effect on DOC-induced apoptosis (data not shown).

DISCUSSION

DPI is used sometimes as a molecular tool with the sole intention of inhibiting NADPH oxidase in cells, so that the role of NADPH oxidase in mediating signal transduction and other cellular processes can be clarified. A concentration of 10 μM DPI has been commonly chosen for particular experiments in numerous cellular studies, such as those reported by other workers in just citing a few [18-22]. Some cellular studies have included experiments using 20- 25 μM DPI [23,24] and even as high as 50 μM DPI [25,26]. Therefore, DPI concentrations spanning this broad range were evaluated in the experiments of the present study. It is thought that DPI inhibits NADPH oxidase by reacting covalently with reduced FAD and/or reduced heme, thereby blocking the ability of such a reduced redox centre to function in electron transfer during enzyme catalysis [27]. This reactivity of DPI likely explains the non-specificity of DPI in inhibiting flavoenzymes besides NADPH

oxidase, such as nitric oxide synthase [28], and both NADPH-cytochrome P450 reductase and NADPH-cytochrome c reductase [29]. Thus, DPI can affect cellular processes unrelated to NADPH oxidase. DPI also inhibits the production of O_2•– and H_2O_2 by mitochondria [6], although DPI seems to stimulate ROS production as well, as discussed further below. Recently, it was found that DPI inhibits the pentose phosphate pathway and the tricarboxylic acid cycle [9]. In the present study, other unusual effects of DPI have been identified.

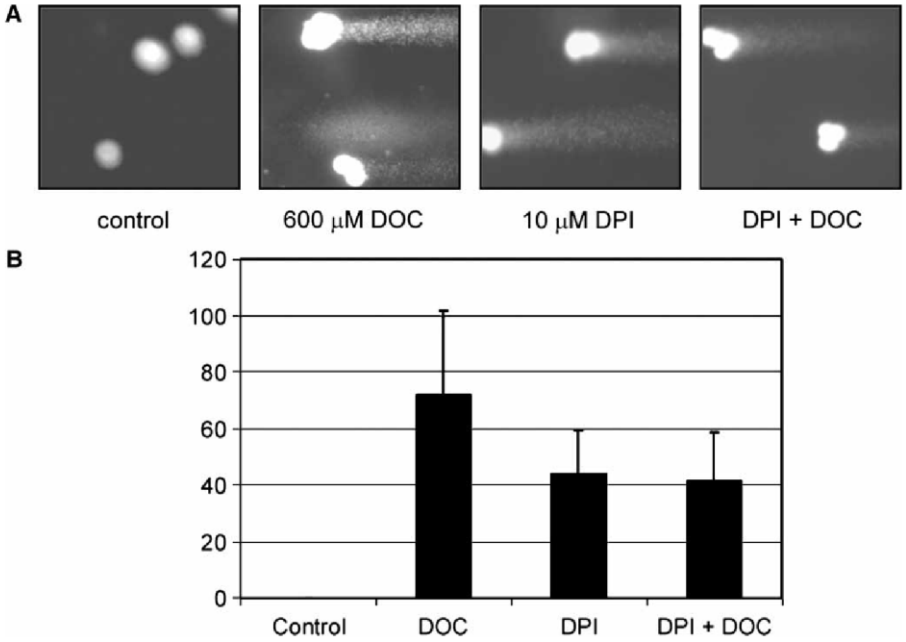

Figure 4. DPI attenuates DOC-induced DNA damage. HCT-116 cells were exposed to either 600 μM DOC or 10 μM DPI for 3 h, but also pre-treated with 10 μM DPI for 0.5 h and then exposed to 600 μM DOC for 3 h. DNA damage was assessed by the comet assay, in which images (representative of three different experiments) were captured (A) and corresponding comet scores (average ± SEM, $n = 3$) were attained (B).

To our knowledge, this is the first time that it has been reported that DPI adversely affects the structural integrity of DNA. However, it is unknown how DPI causes DNA damage in HCT-116 cells. Nonetheless, it is conceivable that ROS are involved. DPI can induce the formation of H_2O_2 [9]. In the

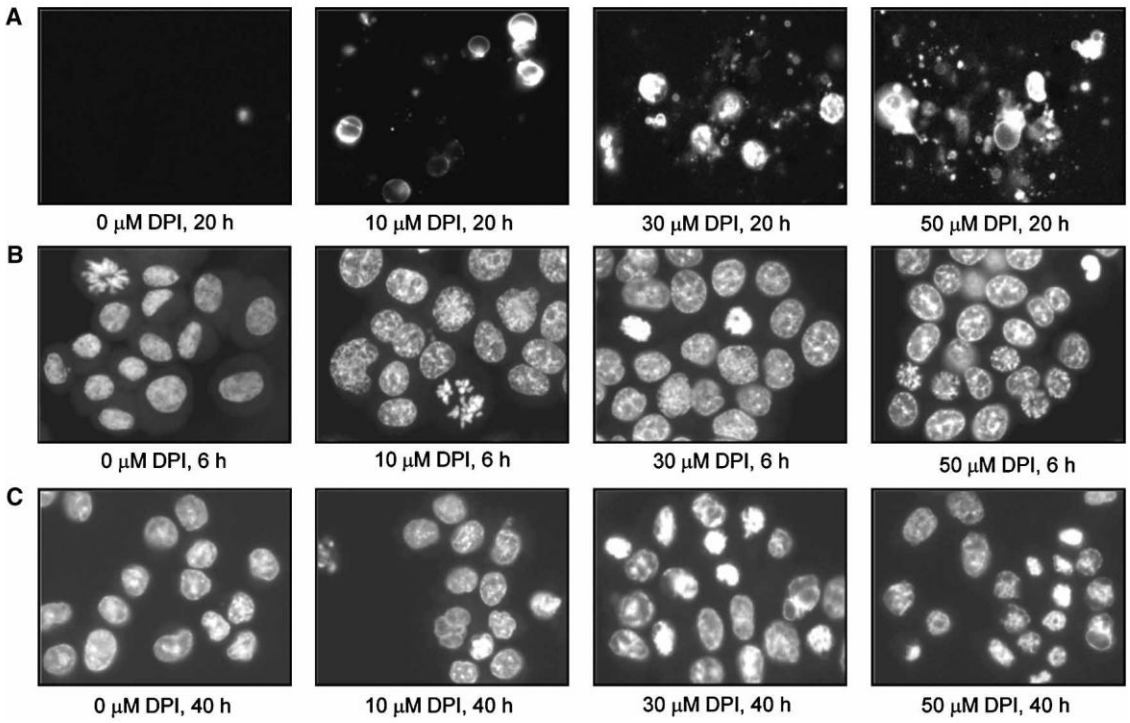

Figure 5. Greater binding of annexin V-Alexa Fluor 488 by DPI-treated cells and presence of chromatin condensation in DPI-treated cells. HCT-116 cells were incubated with 0–50 μM DPI for 20 h. Then, the annexin V-Alexa Fluor 488 binding assay was performed with visualization of the cells by fluorescence microscopy (A). Additionally, HCT-116 cells were incubated with 0–50 μM DPI for either 6 h (B) or 40 h (C). Then, the chromatin in cells was stained with DAPI for examination by fluorescence microscopy. In both assays, the results from three different experiments were similar.

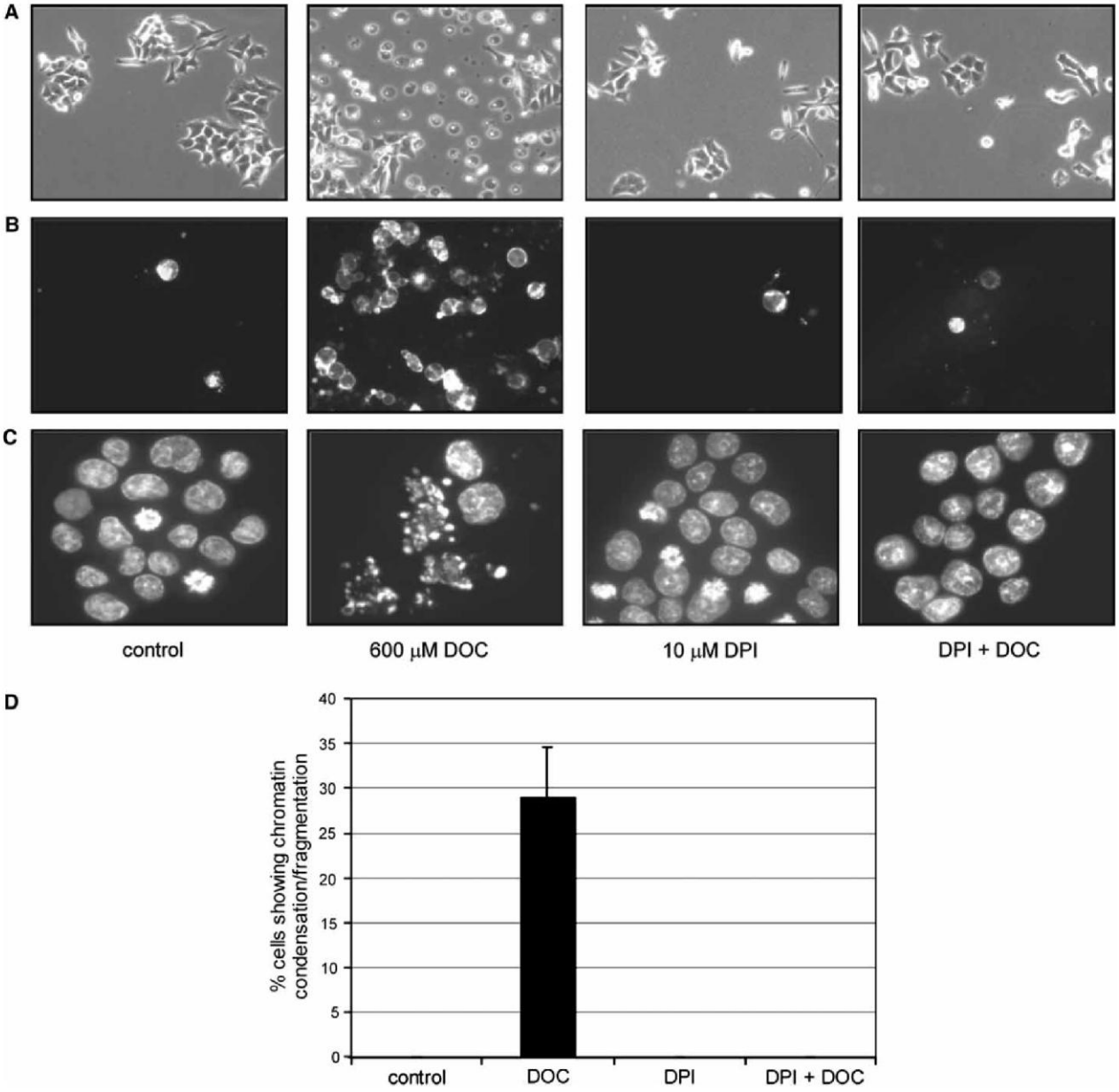

Figure 6. DPI prevents the occurrence of apoptotic events caused by DOC. HCT-116 cells were exposed to either 600 μM DOC or 10 μM DPI for 3 h, but also pre-treated with 10 μM DPI for 0.5 h and then exposed to 600 μM DOC for 3 h. Then, the appearance of the cells was photographed (A). Also, the annexin V-Alexa Fluor 488 binding assay was performed on the cells (B). Furthermore, cells were stained with DAPI to attain the images (C) for subsequent generation of the bar graph (average ± SEM, $n = 3$) showing the percentage of cells with condensed/fragmented chromatin (D). All of the imaging results are representative of three different experiments.

presence of trace amounts of transition metals such as iron, H_2O_2 can be transformed into the hydroxyl radical [30] that in turn is thought to attack the deoxyribose moiety of DNA to cause structural damage [31]. Recently, DPI at a certain concentration was reported to increase the nitration of tyrosine residues in cellular proteins [8]. This finding suggests that DPI can induce the formation of reactive nitrogen species (RNS). RNS are capable of damaging DNA as well [32]. However, the possible involvement of RNS and particularly ROS in DPI-induced DNA damage remains rather unclear, in view of the present findings with antioxidants showing that Tiron, but not NAC, attenuated DPI- induced DNA damage.

In retrospect, based on the concept that DPI can accept an electron from reduced redox centres and become a phenyl radical [27], it may be that such a DPI phenyl radical directly attacks DNA to eventually result in single-strand breaks.

In assessing whether DPI and DOC induce oxidative stress in HCT-116 cells, differences were seen. The data obtained with one chosen fluorescence- based method indicate that DOC increases the formation of ROS, which is consistent with the findings of other workers [10], but that DPI does not increase ROS formation, which is in contrast to what has been found previously but using N11 mouse glial cells and a different method of ROS determination [9]. On the other hand, using another fluorescence-based method but with more specificity, it is shown that DOC, and also DPI to a lesser extent, increases mitochondrial generation of O_2•– in HCT116 cells. Regarding the effect of DPI, opposite results have been reported in unstimulated ML-1-derived monocytes/macrophages [6]. That is, DPI decreased, rather than increased, mitochondrial generation of O_2•–, which was suggested to be due to inhibition of mitochondrial complex I. However, as pointed out in a recent paper [33], previous studies have been inconsistent regarding the ability of DPI to induce ROS formation. This situation is probably a reflection of the different cell types used, experimental conditions (particularly the DPI concentration and incubation time) and the specific procedure utilized to assess ROS formation. Nevertheless, the current finding that DPI increases mitochondrial O_2•– generation in HCT-116 cells is in agreement with at least two other studies [7,34].

In addition to both of them inducing mitochondrial O_2•– generation in HCT-116 cells, DOC and especially DPI each disrupted mitochondrial functioning, that is caused an apparent collapse of MMP. Similar MMP collapse has been reported before in DOC-treated HCT-116 cells [35] and DPI-treated human HL-60 leukaemia cells [7]. Moreover, the present findings that DPI increases mitochondrial O_2•– generation and collapses MMP have also been reported by other workers [7] who suggested that DPI causes these two effects by inhibiting the mitochondrial electron transport chain at complex I.

In examining interactive effects of DPI and DOC under the current set of experimental conditions, a most interesting conclusion can be drawn. Part of the data indicates that DPI largely inhibited DOC-induced ROS formation, which is an inhibitory effect also produced by the antioxidant, Tiron, which scavenges O_2•– but also chelates redox-active transition metals [15] that can initiate formation of downstream ROS [30] . However, it is not entirely clear how DPI decreases the capacity of DOC to induce ROS in general. Other data indicate that DPI could not specifically inhibit DOC-induced mitochondrial O_2•– generation, but it should be noted again that DPI by itself increased mitochondrial O_2•– generation as also found in other studies [7,34]. Furthermore, DPI could not restore the partial loss of MMP caused by DOC, which would not be totally unexpected considering that DPI by itself caused almost total loss of MMP. A previous study [35] showed that DOC-induced loss of MMP was not preventable by rotenone, which like DPI is known to inhibit complex I of the mitochondrial electron transport chain. Moreover, it was shown that rotenone by itself did not cause a loss of MMP, which is in contrast to the effect produced by DPI in the present study.

Cellular DNA damage is often accompanied by increased expression of certain genes, particularly GADD153. For example, exposing cells to DNA- damaging agents such as peroxynitrite [36], UV radiation [37] and anti-cancer drugs [38] cause upregulation of GADD153. These experimental conditions often create a cellular environment resulting in cell death via apoptosis. GADD153 appears to have a direct role in initiating apoptosis, based on the occurrence of apoptosis in GADD153 expression vector-transfected cells [39]. Although DPI induced DNA damage in HCT-116 cells, GADD153 protein was not induced by DPI, at least before the occurrence of the early stages of apoptosis, as would be indicated by membrane phospholipid translocation, suggesting that GADD153 does not play a role in the apoptosis caused by DPI. In any event, the ability of DPI to induce apoptosis in HCT-116 cells is consistent with other studies. DPI caused apoptosis in human umbilical vein endothelial cells [8] and also in HL-60 human promyelocytic leukaemia cells [7] . In both of these previous reports, it was concluded that ROS were involved in the apoptosis initiated by DPI. With respect to DOC, the DNA damage induced by DOC was attenuated by DPI, but DPI did not attenuate the consequent GADD153 mRNA upregulation caused by DOC. In other words, attenuation of DOC-induced DNA damage by DPI was not associated with any reduction in GADD153 mRNA upregulation, which would imply that the increased expression of GADD153 mRNA caused by DOC may not necessarily be the consequence of DNA damage per se.

In view of the ability of DPI to induce DNA damage and apoptosis, it is rather remarkable that DPI largely negated the ability of DOC to induce DNA damage and consequent apoptosis, which is another novel finding in the present study. Since DPI is often considered a NADPH inhibitor, a fundamental interpretation would be that NADPH oxidase is somehow involved in mediating the cytotoxic effect of DOC. After all, it is known that bile acids induce the generation of ROS in cells [10] and a potential source of ROS could be the reaction catalysed by NADPH oxidase. To the contrary, two other common NADPH oxidase inhibitors did not prevent the apoptosis caused by DOC in HCT-116 cells, implying that NADPH oxidase may not actually be involved in the apoptosis caused by DOC. Therefore, future research is needed to investigate the possible involvement of other DPI-inhibitable flavoenzymes in elucidating the ability of DPI to counteract the effects of DOC.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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