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A Novel Mechanism, Uniquely Dependent on Mitochondrial Calcium Accumulation, Whereby Peroxynitrite Promotes Formation of Superoxide/Hydrogen Peroxide and the Ensuing Strand Scission of Genomic DNA

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Abstract

High concentrations of peroxynitrite elicit delayed formation of DNA-damaging species through a mechanism dependent on mitochondrial Ca^{2+} accumulation and inhibition of complex III. A second mechanism, requiring remarkably lower peroxynitrite concentrations, is observed in the presence of *bona fide* complex III inhibitors and is Ca^{2+} independent. We now report evidence for a third mechanism, also operative with low peroxynitrite concentrations, independent of electron transport, and entirely based on mitochondrial Ca^{2+} accumulation. This concept was established by using permeabilized respiration-proficient and -deficient U937 cells supplemented with Ca^{2+}, inhibitors of mitochondrial Ca^{2+} accumulation, and specific respiratory-chain inhibitors. The results obtained were validated by experiments performed with intact cells, by using caffeine (Cf) to promote mitochondrial Ca^{2+} accumulation. Under these conditions, low concentrations of peroxynitrite, otherwise unable to generate detectable DNA cleavage, caused maximal DNA strand scission through a mechanism insensitive to respiratory-chain inhibitors or to the respiration-deficient phenotype. The effects of Cf were mimicked by other ryanodine receptor agonists, were suppressed by ryanodine, and were not observed in cells failing to express the ryanodine receptor, as differentiated U937 cells or human monocytes. This study provides evidence for a novel mechanism whereby peroxynitrite may indirectly mediate DNA strand scission under inflammatory conditions. *Antioxid. Redox Signal.* 13, 745–756.

Introduction

PEROXYNITRITE, the coupling product of superoxide and nitric oxide, is a potent biologic oxidant that can directly damage the DNA *in vitro* [i.e., by using purified DNA preparations (5–7, 19, 32)]. Although direct effects may also take place *in vivo* (23, 24), it would appear that the oxidant promotes mostly indirect events, finally leading to DNA-strand scission in intact cells. This notion is strongly suggested by studies indicating that DNA damage induced by peroxynitrite is blunted by Ca^{2+} chelators (30). The intracellular concentration of the cation is remarkably enhanced by peroxynitrite (30, 31). Additional evidence for the indirect mechanism derives from our studies showing that the DNA damage generated by a bolus of peroxynitrite is barely detectable at 5 min, but then linearly increases with time for $\leq$30 min of incubation (17). As peroxynitrite very rapidly decomposes at physiologic pH values, these results obviously imply the triggering of events leading to the time-dependent formation of secondary DNA-damaging species. We subsequently found that peroxynitrite promotes the formation of superoxide, in a reaction in which ubisemiquinone serves as an electron donor (11, 17). In particular, the mitochondrial formation of superoxide was strictly dependent on an inhibitory effect of peroxynitrite on complex III of the respiratory chain (17), as well as on the mitochondrial accumulation of Ca^{2+} (16), an event associated with the mobilization of the cation mediated by peroxynitrite (31). Additional critical events were represented by the mitochondrial conversion of superoxide ions in H_2O_2, by the migration of the latter to the nucleus, and the formation of the final DNA-damaging species, most likely the hydroxyl radical (11, 16, 17).

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As this mechanism is strictly dependent on mitochondrial Ca^{2+} accumulation and inhibition of electron transport, it will be referred to as Ca^{2+}-/respiratory chain-dependent mechanism (CRDM). Our studies, largely performed in promonocytic U937 cells, also provided information on the source of the cation mobilized by peroxynitrite, essentially represented by the ryanodine receptor (RyR) (16). Inhibition of Ca^{2+} mobilization from this pool was invariably associated with prevention of mitochondrial Ca^{2+} accumulation and of the ensuing Ca^{2+}-dependent events leading to DNA-strand scission.

We recently described an additional mechanism whereby peroxynitrite mediates DNA-strand scission under conditions of enforced inhibition of complex III, entirely dependent on inhibition of electron transport, with hardly any contribution of mitochondrial Ca^{2+} accumulation (11). This mechanism, triggered by low concentrations of peroxynitrite (*e.g.*, 40 μM) in the presence of *bona fide* complex III inhibitors, also involved ubisemiquinone-dependent superoxide formation and will be referred to as respiration-dependent/Ca^{2+}-independent mechanism (RDM). Experiments in progress implicate the involvement of RDM also in the enhancing effects mediated by intracellular ascorbic acid, documented in recent articles from our laboratory (13, 14).

The present study led to the identification of a third mechanism, once again mediated by superoxide formation, entirely dependent on enforced mitochondrial Ca^{2+} accumulation, with hardly any contribution of effects on the respiratory chain. The novel mechanism is referred to as Ca^{2+}-dependent/respiratory chain–independent mechanism (CDM) and appears to be relevant for conditions in which cells are exposed to low levels of peroxynitrite and agents, or conditions, enforcing mitochondrial Ca^{2+} accumulation (*e.g., via* mobilization of the cation from the RyR).

Materials and Methods

Chemicals

Dihydrorhodamine 123 (DHR), MitoSOX red, and Rhod 2-acetoxymethyl ester (AM) were purchased from Molecular Probes (Leiden, The Netherlands). 3-Morpholinosydnonimine (SIN-1), caffeine (Cf), 4-chloro-m-cresol (4-CmC), $CaCl_2$, antimycin A, ryanodine (Ry), ruthenium red (RR), $LaCl_3$, rotenone, myxothiazol, catalase, and the remaining chemicals were from Sigma-Aldrich (Milan, Italy).

Cell-culture and treatment conditions

U937 human myeloid leukemia cells were cultured in suspension in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Euroclone, Celbio Biotecnologie, Milan, Italy), penicillin (100 units/ml), and streptomycin (100 μg/ml) (Euroclone), at 37°C in T-75 tissue-culture flasks (Corning, Corning, NY) gased with an atmosphere of 95% air/5% CO_2.

Respiration-deficient U937 cells were isolated as described in (17). In some experiments, U937 cells were differentiated to monocytes by 4-day growth in culture medium supplemented with 1.3% dimethylsulfoxide (DMSO), as previously described (15).

Human peripheral mononuclear cells were obtained from buffy coats of healthy blood donors through the courtesy of Centro Trasfusionale, Ospedale civile "Fraternità di S. Maria della Misericordia," Urbino, Italy. Human peripheral mononuclear cells were isolated by Ficoll gradient centrifugation, and monocytes were purified by adherence in RPMI medium. Nonadherent cells were removed by repeated washing with phosphate-buffered saline (8 g/L NaCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4, and 0.2 g/L KCl), whereas adherent cells were subsequently scraped with trypsin, and then used for monocyte experiments.

Experiments with intact cells were performed by using 15-ml plastic tubes containing 5×10^5 cells (2 ml) of prewarmed saline A (8.182 g/L NaCl, 0.372 g/L KCl, 0.336 g/L $NaHCO_3$, and 0.9 g/L glucose). Similar conditions were used in experiments with permeabilized cells. Permeabilization was achieved by adding digitonin (10 μM) to a medium consisting of 0.25 *M* sucrose, 0.1% (wt/vol) bovine serum albumin, 10 m*M* $MgCl_2$, 10 m*M* K^+-Hepes, 5 m*M* KH_2PO_4, pH 7.2, at 37°C. Under these conditions, digitonin permeabilizes the plasma membrane but leaves mitochondrial membranes intact (9).

Peroxynitrite, synthesized as previously described (1), was rapidly added on the walls of the plastic tubes and mixed to equilibrate the peroxynitrite concentration on the culture medium. To avoid changes in pH due to the high alkalinity of the peroxynitrite stock solution, an appropriate amount of 1.5N HCl was also added to the walls of the tubes before peroxynitrite. Decomposed peroxynitrite solution and decomposed SIN-1 were generated as previously described (1).

Stock solutions of RR, Cf, $CaCl_2$, $LaCl_3$, and catalase were freshly prepared in saline A. Ry, antimycin A, myxothiazol, and 4-CmC were dissolved in 95% (vol/vol) ethanol. SIN-1 and rotenone were dissolved in DMSO. At the treatment stage, the final ethanol or DMSO concentrations were never higher than 0.05%. Under these conditions, ethanol, or DMSO, was neither toxic nor DNA damaging, nor did it affect the cytogenotoxic properties of peroxynitrite.

Measurement of mitochondrial Ca^{2+}

Dihydro-Rhod 2-AM was freshly synthesized from Rhod 2-AM by using reduction by sodium borohydride (protocol from Molecular Probes). This indicator fluoresces only after it is oxidized to Rhod 2, which occurs preferentially within mitochondria (18). Cells were first exposed for 30 min (4°C) to 5 μM dihydro-Rhod 2-AM, washed 3 times with saline A, and finally incubated for 5 h in RPMI 1640 medium (37°C). This two-step cold loading/warm incubation protocol achieves selective loading of dihydro-Rhod 2-AM into the mitochondria (29). The cells were then washed 3 times with saline A and treated for 10 min, as detailed earlier and in the legend to the figures, in 35-mm tissue culture dishes containing an uncoated coverslip. Under these conditions, U937 cells rapidly attach to the coverslip. Fluorescence images were captured with a BX-51 microscope (Olympus, Milan, Italy), equipped with a SPOT-RT camera unit (Diagnostic Instruments, Delta Sistemi, Rome, Italy). The excitation and emission wavelengths were 540 and 590 nm, respectively, with a 5-nm slit width for both emission and excitation. Images were collected with exposure times of 100–400 ms, digitally acquired, and processed for fluorescence determination at the single-cell level on a personal computer by using Scion Image software (Scion Corp., Frederick, MD). Mean fluorescence values were

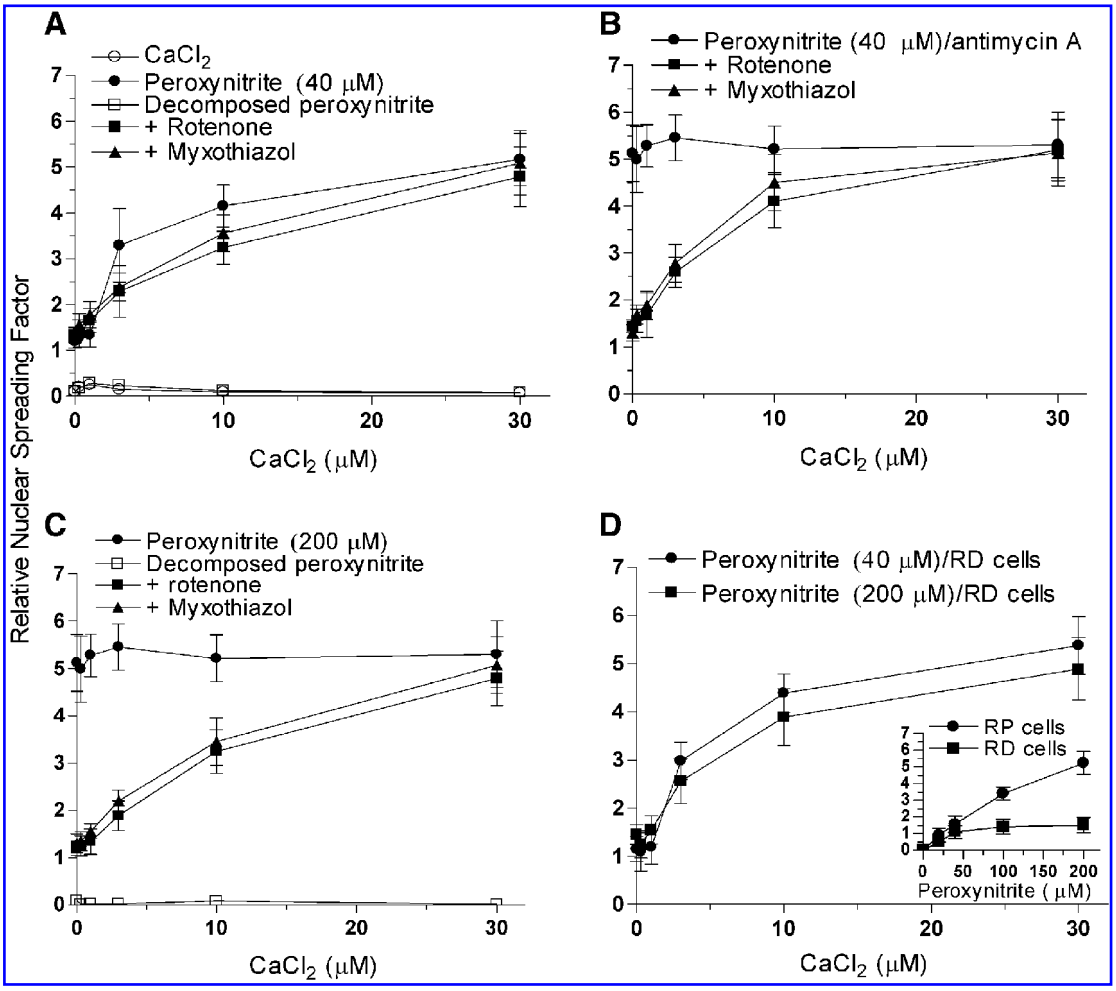

FIG. 1. DNA-strand scission induced by peroxynitrite in permeabilized cells: evidence for a Ca^{2+}-dependent mechanism, unaffected by respiratory chain inhibitors, or by the respiration-deficient phenotype. Permeabilized cells were exposed for 10 min to 40 (**A**) or 200 μM (**C**) peroxynitrite alone (*solid circles*) with 0.5 μM rotenone (*solid squares*), or 5 μM myxothiazol (*solid triangles*), in the presence of increasing concentrations of $CaCl_2$. (**A, C**) The effect of decomposed peroxynitrite (*open squares*), whereas the effect of $CaCl_2$ without any additional manipulation is reported only in (**A**) (*open circles*). In other experiments (**B**), permeabilized cells were exposed for 10 min to 40 μM peroxynitrite/1 μM antimycin A alone (*solid circles*) or associated with rotenone (*solid squares*), or myxothiazol (*solid triangles*), in the presence of increasing concentrations of $CaCl_2$. Permeabilized respiration-deficient (RD) cells (**D**) were exposed for 10 min to 40 (*solid circles*) or 200 μM (*solid squares*) peroxynitrite in the presence of increasing concentrations of $CaCl_2$. The inset shows the level of DNA single-strand breaks caused by increasing concentrations of peroxynitrite in respiration-proficient (RP, *open circles*) or RD (*solid squares*) cells. After treatments, cells were analyzed for DNA damage, as detailed in Materials and Methods. Results represent the mean ± SD calculated from three to five separate experiments.

determined by averaging the fluorescence values of ≥least 50 cells per treatment condition/experiment.

Measurement of DNA single-strand breakage by the alkaline halo assay

DNA single-strand breakage was determined by using the alkaline halo assay developed in our laboratory (3). It is important to keep in mind that, although we refer to DNA-strand scission throughout the text, the DNA nicks measured by this technique under alkaline conditions may include alkali-labile sites in addition to direct strand breaks. Details on the alkaline halo assay and processing of fluorescence images and on the calculation of the experimental results are also given in (3). DNA damage was quantified by calculating the nuclear-spreading factor value, representing the ratio between the area of the halo (obtained by subtracting the area of the nucleus from the total area, nucleus + halo) and that of the nucleus, from 50 to 75 randomly selected cells/experiment/treatment condition. Results are expressed as relative nuclear spreading-factor values calculated by subtracting the nuclear spreading-factor values of control cells from those of treated cells.

DHR and MitoSOX red oxidation

Cells were first exposed for 3 min to peroxynitrite in 35-mm tissue-culture dishes containing an uncoated coverslip and incubated for a further 27 min in the presence of 10 μM DHR. In other experiments, the cells were first exposed for 15 min

(37°C) to 5 μM MitoSOX red, washed twice with saline A, and finally treated as detailed in the legends to the figures. After treatments, the cells were washed twice and analyzed with a fluorescence microscope.

The resulting images were taken and processed as described earlier. The excitation and emission wavelengths were 488 and 515 nm (DHR), and 510 and 580 nm (MitoSOX red), with a 5-nm slit width for both emission and excitation. Mean fluorescence values were determined by averaging the fluorescence values of $\geq$50 cells/treatment condition/experiment.

Aconitase activity

U937 cells were washed twice with saline A, resuspended in the same solution at a density of 5×10^6 cells/20 ml, and treated with peroxynitrite. After treatments, the cells were washed twice with saline A and finally sonicated thrice on ice with a Branson sonifier operating at 20 W for 15 s. The resulting homogenates were centrifuged for 5 min at 18,000 *g* at 4°C. Aconitase activity was determined spectrophotometrically in the supernatant, as described by Gardner (10).

Statistical analysis

The results are expressed as mean $\pm$ SD. Statistical differences were analyzed by one-way ANOVA followed by Dunnett's test for multiple comparison or two-way ANOVA followed by Bonferroni's test for multiple comparison. A value of $p < 0.05$ was considered significant.

Results

DNA-strand scission induced by peroxynitrite in permeabilized cells: preliminary evidence for a novel Ca^{2+}-dependent/respiratory chain–independent mechanism

The results illustrated in Fig. 1A confirm our recent findings (16), indicating that permeabilized cells exposed to 40 μM peroxynitrite progressively accumulate DNA single-strand breaks in the presence of increasing concentrations of $CaCl_2$, with a maximal response being observed at 30 μM. Ca^{2+} did not produce effects in the absence of peroxynitrite, and the oxidant produced very low level of breaks in the absence of reagent $CaCl_2$. Furthermore, the enhancing effects of Ca^{2+} were not observed by using decomposed peroxynitrite. Interestingly, DNA-strand scission mediated under these conditions was insensitive to the complex I inhibitor rotenone (0.5 μM), which, however, abolished the DNA cleavage elicited by the same concentration of peroxynitrite in cells supplemented with the complex III inhibitor antimycin A (1 μM, Fig. 1B), or with 200 μM peroxynitrite (Fig. 1C), producing maximal DNA-strand breakage in the absence of additional treatments (11, 16, 17). No effect was observed by using decomposed peroxynitrite. Interestingly, exogenous Ca^{2+} circumvented the inhibitory effects of rotenone, and the resulting curves for DNA-strand scission (Fig. 1B and C) were identical to the one obtained with 40 μM peroxynitrite alone (Fig. 1A). Conversely, Ca^{2+} failed to affect maximal DNA-strand scission induced by peroxynitrite/antimycin A (Fig. 1B) or by the high peroxynitrite concentration (Fig. 1C). Similar results were obtained in all of these experiments by replacing rotenone with myxothiazol (5 μM), an inhibitor of the electron flow from the reduced coenzyme Q to cytochrome c_1 (2).

Experiments were next performed with respiration-deficient U937 cells, with the aim of reproducing these effects without the use of respiratory-chain inhibitors. These cells, although resistant to the effects of concentrations of peroxynitrite promoting DNA-strand scission in the respiration-proficient counterpart (inset to Fig. 1D), displayed collateral sensitivity to the Ca^{2+}-dependent DNA cleavage induced by peroxynitrite (Fig. 1D), a response obviously unaffected by rotenone or myxothiazol (not shown). It is interesting to note that the curves obtained with 40 or 200 μM peroxynitrite were once again identical.

These results suggest the existence of a novel Ca^{2+}-dependent mechanism, independent of electron transport in the mitochondrial respiratory chain, and therefore different from CRDM (mediating DNA damage induced by 200 μM peroxynitrite) and RDM (mediating DNA damage induced by 40 μM peroxynitrite/antimycin A). From these results, we also conclude that CDM, as previously reported for RDM (16), is maximally induced at 40 μM peroxynitrite.

DNA cleavage induced by peroxynitrite via the Ca^{2+}-dependent/respiratory chain–independent mechanism in permeabilized cells: a role for mitochondrial Ca^{2+} accumulation and for the ensuing H_2O_2 formation

The result reported in Table 1 are from experiments testing the effects of RR (200 n*M*, a concentration specifically preventing mitochondrial Ca^{2+} uptake) (4), lanthanium ions (100 μM, known to inhibit competitively Ca^{2+} uptake) (26), and enzymatically active (or heat-inactivated) catalase (10 Sigma Units/ml) on the DNA-strand scission induced by 40 μM peroxynitrite/30 μM $CaCl_2$ (putative CDM), 40 μM peroxynitrite/antimycin A (RDM), or 200 μM peroxynitrite (CRDM). The effect of 40 μM peroxynitrite/30 μM $CaCl_2$ also was tested in respiration-deficient cells, which, based on the classification made in the previous section, qualifies as CDM. The results obtained are consistent with the notion that, as previously shown for CRDM (16) and RDM (11), CDM-dependent DNA damage is sensitive to enzymatically active catalase and thus attributable to H_2O_2. Importantly, however, the process mediating H_2O_2 formation in CDM, as in CRDM, but unlike that in RDM, appears to take place through a mechanism requiring mitochondrial Ca^{2+} accumulation.

DNA cleavage induced by peroxynitrite through the Ca^{2+}-/respiratory chain–dependent mechanism requires less Ca^{2+} than the Ca^{2+}-dependent/respiratory chain–independent mechanism

We performed experiments to investigate the Ca^{2+} requirements for CRDM. In these experiments, permeabilized cells were exposed to 200 μM peroxynitrite in the presence of Ry, which, by blocking the mobilization of Ca^{2+} from the RyR, prevents the mitochondrial accumulation of the cation and the ensuing formation of species leading to strand scission of genomic DNA (16). Ry prevented the DNA-strand scission mediated by peroxynitrite, and the DNA-damaging response was promptly reestablished by exogenous Ca^{2+} (Fig. 2A). Under these conditions, the maximal effects are observed by using 3 μM $CaCl_2$, a concentration one order of magnitude

Table 1. DNA Cleavage Induced by Peroxynitrite in Permeabilized Cells *via* the Ca^{2+}-dependent/ Respiratory Chain-independent Mechanism Requires Mitochondrial Ca^{2+} Accumulation and Is Mediated by H_2O_2

Treatment	*Relative nuclear spreading factor*
Respiration-proficient cells	
Peroxynitrite (40 μM)/$CaCl_2$ (30 μM)	5.23 ± 0.65
+ RR	1.41 ± 0.35*
+ $LaCl_3$	1.24 ± 0.27*
+ Catalase	1.31 ± 0.40*
+ Boiled catalase	5.29 ± 0.33
Peroxynitrite (40 μM)/antimycin A (1 μM)	5.65 ± 0.49
+ RR	5.39 ± 0.56
+ $LaCl_3$	5.27 ± 0.64
+ Catalase	1.28 ± 0.21*
+ Boiled catalase	5.31 ± 0.47
Peroxynitrite (200 μM)	5.02 ± 0.50
+ RR	1.41 ± 0.23*
+ $LaCl_3$	1.29 ± 0.28*
+ Catalase	1.19 ± 0.37*
+ Boiled catalase	4.99 ± 0.62
Peroxynitrite (40 μM)/Cf (10 mM)	5.02 ± 0.50
+ Ry	1.35 ± 0.34*
+ RR	1.41 ± 0.23*
+ $LaCl_3$	1.29 ± 0.27*
+ Rotenone	5.19 ± 0.56
+ Myxothiazol	5.95 ± 0.48
+ Catalase	1.19 ± 0.27*
+ Boiled catalase	4.99 ± 0.27
Respiration-deficient cells	
Peroxynitrite (40 μM)/$CaCl_2$ (30 μM)	5.11 ± 1.00
+ RR	1.36 ± 0.48*
+ $LaCl_3$	1.29 ± 0.37*
+ Catalase	1.35 ± 0.30*
+ Boiled catalase	5.23 ± 0.61
Peroxynitrite (40 μM)/Cf (10 mM)	5.07 ± 0.55
+ Ry	1.19 ± 0.30*
+ RR	1.25 ± 0.41*
+ $LaCl_3$	1.37 ± 0.37*
+ Rotenone	5.36 ± 0.32
+ Myxothiazol	4.95 ± 0.58
+ Catalase	1.28 ± 0.21*
+ Boiled catalase	5.31 ± 0.49*

Permeabilized cells were exposed for 10 min to 40 or 200 μM peroxynitrite associated with 30 μM $CaCl_2$, 1 μM antimycin A, or 10 mM Cf, in the absence or presence of 20 μM Ry, 200 nM RR, 100 μM $LaCl_3$, 0.5 μM rotenone, 5 μM myxothiazol, or 10 Sigma Units/ml catalase (enzymatically active or heat inactivated). The level of DNA single-strand breaks was measured by the alkaline halo assay immediately after the treatments.

The relative nuclear spreading factor values represent the mean ± SD calculated from three to five separate experiments. *$p < 0.001$ as compared with cells exposed to peroxynitrite alone or associated with $CaCl_2$, antimycin A, or Cf (one-way ANOVA followed by Dunnett's test).

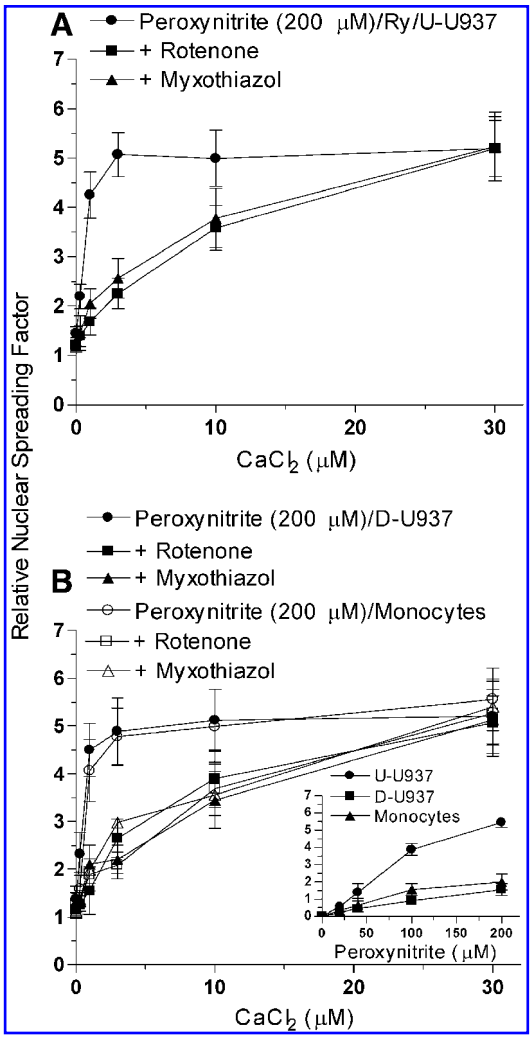

FIG. 2. Concentration dependence for Ca^{2+} in the DNA-strand scission induced by peroxynitrite through the respiratory chain–dependent and –independent mechanisms. (**A**) Permeabilized undifferentiated -U937 (U-U937) cells were exposed for 10 min to 200 μM peroxynitrite/20 μM Ry alone (*solid circles*), or associated with rotenone (*solid squares*), or myxothiazol (*solid triangles*), in the presence of increasing concentrations of $CaCl_2$. (**B**) Permeabilized DMSO-differentiated U937 (D-U937) cells (*solid symbols*), or human monocytes (*open symbols*), were exposed for 10 min to 200 μM peroxynitrite alone (*circles*), or associated with rotenone (*squares*), or myxothiazol (*triangles*), in the presence of increasing concentrations of $CaCl_2$. The inset shows the level of DNA single-strand breaks caused by increasing concentrations of peroxynitrite in U-U937 cells (*solid circles*), D-U937 cells (*solid squares*), or human monocytes (*solid triangles*). After treatments, cells were analyzed for DNA damage, as detailed in Materials and Methods. Results represent the mean ± SD calculated from three to five separate experiments.

smaller than that required for the maximal response through CDM paradigms described in Fig. 1A–D. This observation implies that 3 μM $CaCl_2$ restores CRDM under conditions in which the latter is abolished through prevention of Ca^{2+} mobilization from the RyR. The contribution of effects on electron transport in the respiratory chain in cells treated as indicated earlier is emphasized by the sensitivity to rotenone or myxothiazol (Fig. 2A). In addition, DNA-strand scission elicited by 200 μM peroxynitrite/Ry/3 μM $CaCl_2$ was sensitive to RR, lanthanium ions, and enzymatically active catalase (not shown).

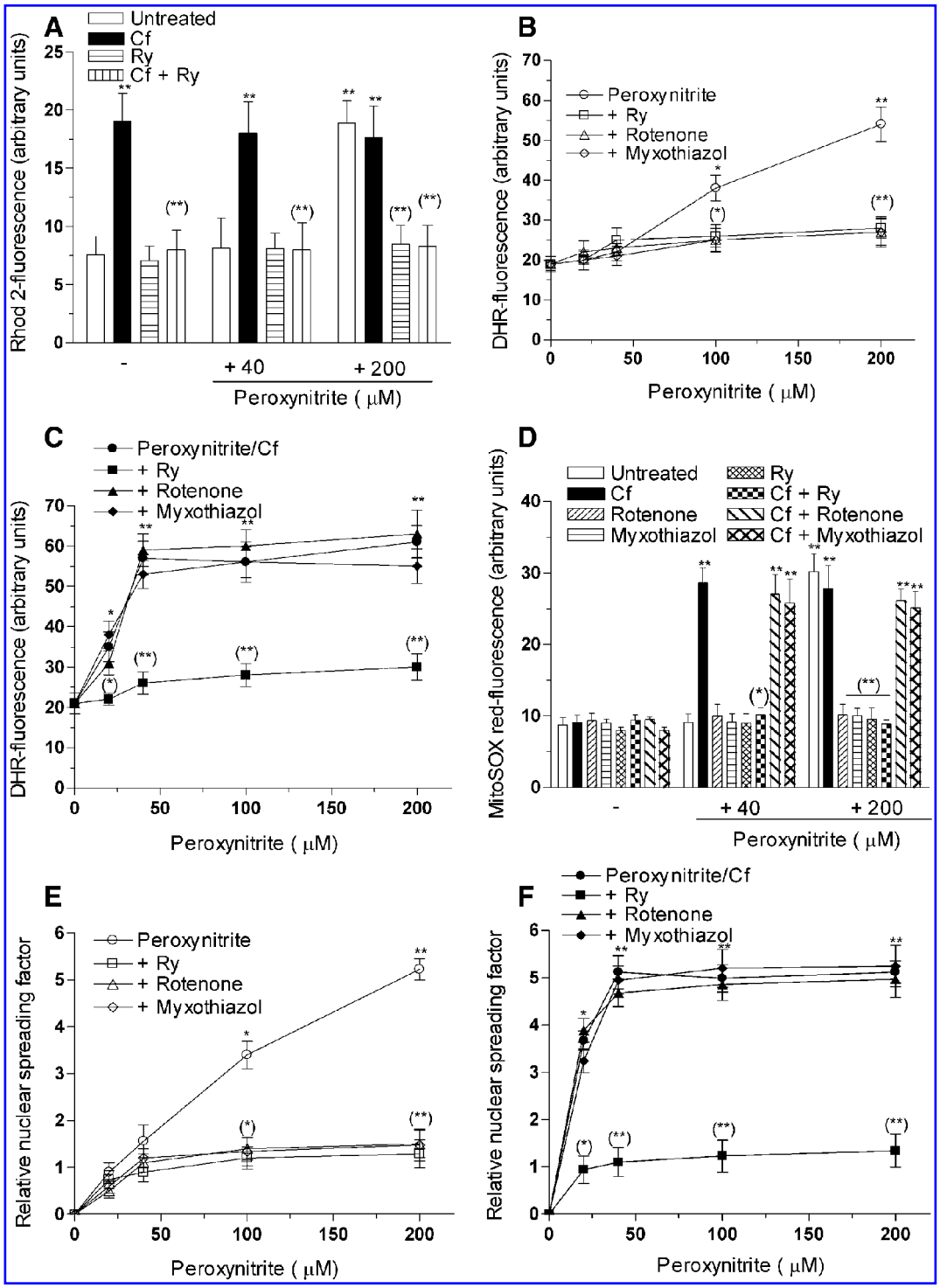

FIG. 3. Peroxynitrite-dependent DNA strand scission mediated by the Ca^{2+}-dependent/respiratory chain–independent mechanism in intact cells. (**A**) Dihydro-Rhod 2-AM preloaded cells were sequentially exposed for 5 min to the vehicle or Ry, for an additional 5 min to 0 or 10 m*M* Cf and finally incubated for a further 10 min with peroxynitrite (40 or 200 μM). Fluorescence was then quantified as detailed in Materials and Methods. (**B–E**) The cells were treated as indicated in the figures and subsequently analyzed for DHR- (**B, C**) or MitoSOX red-fluorescence (**D**), or DNA damage (**E, F**), as detailed in Materials and Methods. Results represent the mean ± SD calculated from three to five separate experiments. *$p < 0.01$; **$p < 0.001$ as compared with untreated cells; (*)$p < 0.01$; (**)$p < 0.001$ as compared with cells treated with Cf, or peroxynitrite alone, or peroxynitrite associated with Cf (ANOVA followed by Dunnett's test).

Table 2. CF Fails to Promote Mitochondrial Ca^{2+} Accumulation and Delayed Superoxide Formation, as well as the Ensuing DNA Strand Scission, in DMSO-Differentiated U937 Cells and Human Monocytes Exposed to Peroxynitrite

Treatment	*Rhod 2-fluorescence (arbitrary units)*	*MitoSOX red-fluorescence (arbitrary units)*	*Nuclear spreading factor*
DMSO-differentiated U937 cells			
Untreated	7.73 ± 1.35	9.85 ± 1.55	0.85 ± 0.26
Cf	7.91 ± 1.47	10.1 ± 1.39	1.01 ± 0.43
Peroxynitrite (40 μM)	9.93 ± 1.42	1.38 ± 0.49	8.99 ± 1.72
+ Cf	8.46 ± 2.01	9.68 ± 1.61	1.40 ± 0.30
Human monocytes			
Untreated	7.04 ± 1.28	8.89 ± 1.56	0.72 ± 0.15
Cf	7.58 ± 2.45	8.16 ± 2.01	0.98 ± 0.32
Peroxynitrite (40 μM)	7.89 ± 1.95	1.19 ± 0.38	8.02 ± 1.84
+ Cf	7.38 ± 2.06	9.67 ± 1.33	1.27 ± 0.25

Cells were exposed for 10 (Rhod 2-fluorescence) or 30-min (MitoSOX red-fluorescence and DNA strand scission) to 0 or 40 μM peroxynitrite, with or without prior addition of Cf. After treatments, the cells were processed as indicated in Materials and Methods. Results represent the mean ± SD from four separate experiments.

On the basis of these findings, the mechanism whereby 200 μM peroxynitrite/Ry/3 μM $CaCl_2$ causes DNA-strand scission qualifies as CRDM. It is interesting to note that the use of 30 μM in the place of 3 μM $CaCl_2$ causes a change in the mechanism of DNA-strand scission, which lost its sensitivity to rotenone or myxothiazol (Fig. 2A), thereby classifying as CDM. More generally, it can be stated that CDM accounts for DNA-strand scission mediated under all of the previously mentioned conditions in which cells are supplemented with exogenous Ca^{2+} and rotenone (or myxothiazol). The resulting curves for DNA-strand scission are superimposable on those shown in Fig. 1, qualifying for CDM.

The outcome of experiments using DMSO-differentiated U937 cells, or primary human monocytes, which do not express the RyR (12), was in line with these findings. These cells were resistant to DNA-strand scission induced by peroxynitrite (inset to Fig. 2B), and the Ca^{2+}-dependent DNA cleavage (sensitive to RR, lanthanium ions, and catalase, not shown) was insensitive to rotenone (or myxothiazol) and superimposable on that obtained with undifferentiated U937 cells supplemented with Ry (Fig. 2A).

From these findings, we conclude that the same peroxynitrite concentration may promote DNA damage through CRDM or CDM, depending on the availability of Ca^{2+}. CRDM requires much less Ca^{2+} than CDM, and the latter prevails over CRDM at high Ca^{2+} concentrations.

DNA cleavage induced by peroxynitrite through the Ca^{2+}-dependent/respiratory chain–independent mechanism: evidence in intact cells

A high concentration of Cf (10 mM) was used to promote mobilization of Ca^{2+} from the RyR associated with the mitochondrial clearance of the cation immediately before addition of increasing concentrations of peroxynitrite. Figure 3A shows that, unlike 40 μM peroxynitrite, Cf significantly enhances the fluorescence response of cells preloaded with dihydro-Rhod 2-AM, thereby providing evidence of mitochondrial Ca^{2+} accumulation. The Rhod 2–derived fluorescence was found to colocalize with the fluorescence of the mitochondrial probe MitoTracker Green (not shown), was not affected by the addition of 40 μM peroxynitrite, and was sensitive to Ry. As previously reported (16), 200 μM peroxynitrite significantly enhanced mitochondrial Ca^{2+} accumulation through an Ry-sensitive mechanism. In line with the notion that peroxynitrite mobilizes Ca^{2+} from the RyR is the observation that the effects of the oxidant were not further enhanced by Cf.

These results indicate that peroxynitrite mobilizes Ca^{2+} from the RyR and validate the use of Cf to enforce the mitochondrial Ca^{2+} accumulation in cells exposed to concentrations of peroxynitrite otherwise failing to generate detectable effects, in absence of additional treatments. Under these conditions, Cf caused a Ry-sensitive leftward shift in the dose–response curves for DHR oxidation (Fig. 3B and C) and DNA-strand scission (Fig. 3E and F), with the maximal effects being observed with 40 μM peroxynitrite. It is important to note that DHR-oxidation studies involved addition of the fluorescent probe 3 min after peroxynitrite (see Methods section), thereby ruling out the possibility of direct oxidation. The notion that peroxynitrite-dependent delayed DHR oxidation is entirely attributable to mitochondrial H_2O_2 was established in recent studies (16, 27, 28). We also performed experiments by using MitoSOX red, a fluorogenic dye selective for the detection of superoxide, the immediate precursor of H_2O_2, in the mitochondria of live cells (22). The results obtained in these experiments are in line with those obtained by using DHR, as the fluorescence response mediated by 200 μM peroxynitrite was in both circumstances suppressed by rotenone or myxothiazol (Fig. 3B and D). More generally, it can be stated that each of the experiments performed with these fluorescent probes produced identical outcomes.

Consistent with an involvement of Ca^{2+} mobilization from the RyR in the enhancing effects of Cf is the observation that the latter fails to promote Rhod 2- or MitoSOX red–derived fluorescence and DNA damage in DMSO-differentiated U937 cells, or in human monocytes (Table 2). These cells do not express the RyR (12) and were indeed resistant to the effects mediated by 200 μM peroxynitrite (not shown), as well as to the enhancing effects of Cf, which failed to promote mitochondrial Ca^{2+} accumulation and the peroxynitrite (40 μM)-dependent MitoSOX red fluorescence response associated with DNA-strand scission (Table 2). As previously shown (Fig. 2B), however, permeabilized DMSO-differentiated U937

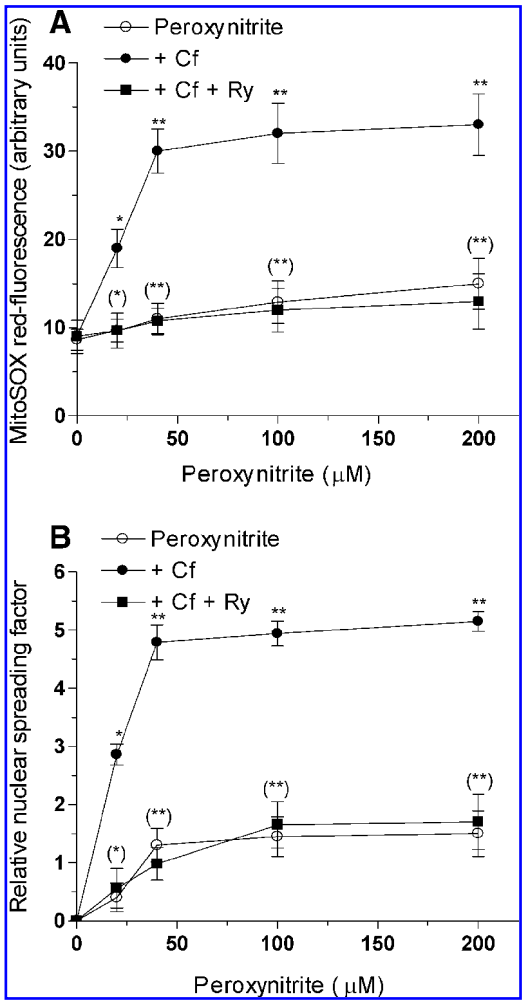

FIG. 4. Cf promotes peroxynitrite-dependent superoxide formation and DNA-strand scission in respiration-deficient cells. (**A, B**) Respiration-deficient cells were first exposed for 5 min to the vehicle (*solid circles*), or Ry (*solid squares*), subsequently incubated for 5 min with Cf, and finally treated for a further 30 min with increasing concentrations of peroxynitrite. In some experiments, the cells were exposed to the sole peroxynitrite (*open circles*). After treatments, the cells were analyzed for MitoSOX red-fluorescence (**A**) and DNA damage (**B**), as detailed in Materials and Methods. Results represent the mean ± SD calculated from three to five separate experiments. *$p < 0.01$; **$p < 0.001$ as compared with untreated cells; (*)$p < 0.01$; (**)$p < 0.001$, as compared with cells treated with peroxynitrite associated with Cf (ANOVA followed by Dunnett's test).

cells, or human monocytes, acquired susceptibility to DNA damage on supplementation of exogenous Ca^{2+}.

Cf remarkably also enhanced the DNA-strand scission induced by peroxynitrite (40 μM) in permeabilized U937 cells, and this response was abolished by Ry, RR, $LaCl_3$ and enzymatically active catalase (Table 1). Interestingly, rotenone and myxothiazol failed to prevent the DNA damage induced under these conditions, in keeping with the notion that CDM mediates the enhancing effects of Cf in the permeabilized cell system. An identical conclusion is reached from experiments performed with intact cells. We found that DHR (Fig. 3C) and MitoSOX red fluorescence (Fig. 3D) responses, as well as DNA-strand scission (Fig. 3F) induced by low concentrations of peroxynitrite in the presence of Cf, although suppressed by Ry, are insensitive to rotenone or myxothiazol. As expected (16), however, the same effects mediated by 100–200 μM peroxynitrite, in the absence of additional treatments, were suppressed by Ry as well as by rotenone and myxothiazol (Fig. 3B and E).

Experiments were next performed in respiration-deficient cells, in which peroxynitrite alone fails to produce mitochondrial H_2O_2 and DNA cleavage (11, 17). We repeated these experiments and found that a functional respiratory chain is not required for the enhancing effects of Cf on both superoxide formation (Fig. 4A) and DNA-strand scission (Fig. 4B). Respiration-proficient (Fig. 3D and F) and -deficient cells (Fig. 4A and B) were indeed equally sensitive to the cocktail peroxynitrite/Cf, and Ry suppressed these responses in both cell types. Identical results were obtained in respiration-deficient/proficient permeabilized cells exposed to 40 μM peroxynitrite/Cf (Table 1). Under both conditions, the DNA damage was suppressed by Ry, RR, lanthanium ions, and enzymatically active catalase, and was insensitive to rotenone or myxothiazol.

A final series of experiments was performed with the aim of further establishing the specificity of the effects mediated by peroxynitrite and Cf in intact cells. We found that 40 μM peroxynitrite, Cf, or the two treatments combined fail to produce toxicity, as measured by the trypan blue exclusion assay (not shown). In addition, identical proliferation rates were detected over 72 h of growth of untreated cells or cells receiving these treatments (not shown). We also found that 4-CmC, another agonist of the RyR (33), mimics the enhancing effects of Cf and produces responses once again sensitive to Ry (Table 3). We then performed experiments in which SIN-1, a well-established peroxynitrite donor (31), was used in the place of authentic peroxynitrite. As indicated in Table 4, we were able to select concentrations of SIN-1, namely 0.1 and 1 mM, producing responses superimposable on those obtained with 40 or 200 μM peroxynitrite with or without Cf and Ry. These effects were not observed by using decomposed SIN-1.

These results demonstrate that Cf enforces mitochondrial Ca^{2+} accumulation and promotes superoxide/H_2O_2 formation and DNA-strand scission in cells exposed to concentrations of peroxynitrite otherwise failing to generate detectable effects in the absence of additional treatments. The results reported in Table 5 also provide evidence of inhibition of aconitase activity, commonly considered a reliable marker of mitochondrial/cytoplasmic superoxide formation. We first established that ~70% of aconitase activity, as in most cell types, is associated with the mitochondrial fraction of U937 cells. Second, we determined that the cocktail antimycin A/peroxynitrite (40 μM) inhibits aconitase activity through a rotenone-sensitive, albeit Ry-insensitive mechanism. Third, we showed that identical effects are obtained by replacing antimycin A with Cf, except that the response obtained acquired sensitivity to Ry and lost sensitivity to rotenone. Hence, the scenario resulting from experiments measuring a sensitive target of superoxide is also compatible with an effect of Cf mediated by CDM.

Collectively, the results described in this section demonstrate that CDM mediates superoxide/H_2O_2-dependent effects, as DNA-strand scission or inhibition of aconitase activity, in intact cells exposed to low concentrations of per-

Table 3. 4-CmC Promotes RY-sensitive Mitochondrial Ca^{2+} Accumulation and Triggers RY-sensitive Superoxide Formation and DNA-strand Scission in U937 Cells Exposed to Peroxynitrite

Treatment	*Rhod 2-fluorescence (arbitrary units)*	*MitoSOX red-fluorescence (arbitrary units)*	*Nuclear spreading factor*
Untreated	7.09 ± 1.13	8.59 ± 1.12	0.98 ± 0.39
4-CmC	18.3 ± 2.25*	9.34 ± 1.89	1.07 ± 0.55
+ Ry	9.06 ± 1.03(*)	10.7 ± 2.34	0.83 ± 0.92
Peroxynitrite (40 μM)	7.89 ± 2.58	8.97 ± 1.08	1.48 ± 0.34
+ 4-CmC	19.4 ± 2.56*	32.4 ± 3.56*	6.28 ± 0.22*
+ Ry	8.23 ± 1.91(*)	11.1 ± 2.04(*)	2.15 ± 0.22(*)

U937 cells were exposed for 10 (Rhod 2-fluorescence) or 30 min (MitoSOX red-fluorescence and DNA-strand scission) to 0 or 40 μM peroxynitrite, with or without prior addition of Ry and/or 4-CmC. After treatments, the cells were processed as indicated in Materials and Methods. Results represent the mean ± SD from four separate experiments.

*$p < 0.001$ as compared with untreated cells.

(*)$p < 0.001$ as compared with 4-CmC alone or associated with peroxynitrite (one-way ANOVA followed by Dunnett's test).

oxynitrite and agents causing mitochondrial Ca^{2+} accumulation.

Discussion

Peroxynitrite indirectly promotes strand scission of genomic DNA through a mechanism (CRDM) associated with mitochondrial Ca^{2+} accumulation and inhibition of electron transport at the level of complex III (16). Under these conditions, superoxide anions are readily generated, in a reaction in which ubisemiquinone serves as an electron donor, and immediately converted to H_2O_2 that, on interaction with divalent iron, promotes the formation of hydroxyl radicals, the putative DNA-damaging species (16). We recently reported the existence of a second important mechanism (RDM) causing DNA cleavage in response to otherwise nongenotoxic concentrations of peroxynitrite (*e.g.*, 40 μM) and *bona fide* complex III inhibitors (11). The DNA-damaging species were again represented by mitochondrial superoxide/H_2O_2, but generated *via* a Ca^{2+}-independent mechanism (11).

These findings are therefore consistent with the notion that specific conditions may trigger DNA-strand scission, even in the presence of low concentrations of peroxynitrite. Recent results obtained in our laboratory implicate RDM in the enhancing effects mediated by ascorbate preloading on secondary H_2O_2 formation and DNA-strand scission induced by peroxynitrite. In our opinion, 40 μM should be considered a low concentration of peroxynitrite that cannot be compared with the remarkably lower levels endogenously detected, because most of the oxidant is degraded extracellularly, when added to the cultures as a bolus; furthermore, in this second condition, the effects are confined to a fraction of a second, whereas formation of endogenous peroxynitrite proceeds for longer intervals. Most important, however, 40 μM should be considered a low concentration of peroxynitrite, as it fails to generate detectable effects in the absence of additional manipulations.

The results presented in this study provide evidence for a novel mechanism (CDM), also mediated by low concentrations of peroxynitrite under conditions of enforced mitochondrial Ca^{2+} accumulation. Once again, the DNA-damaging species are represented by mitochondrial superoxide/H_2O_2, generated through a mechanism independent of electron transport and entirely based on mitochondrial Ca^{2+} accumulation. Various potential respiratory chain–independent mitochondrial sources of superoxide exist (8), as reduced flavins and flavoproteins (8, 21) and the α-ketoglutarate dehydrogenase complex (8, 25), a Ca^{2+}-dependent mitochondrial

Table 4. Effects of CF Preloading in Intact U937 Cells Exposed to SIN-1

Treatment	*Rhod 2-fluorescence (arbitrary units)*	*MitoSOX red-fluorescence (arbitrary units)*	*Nuclear spreading factor*
Untreated	7.98 ± 1.30	9.95 ± 1.35	0.81 ± 0.16
SIN-1 (0.1 mM)	8.09 ± 1.56	10.9 ± 1.45	0.99 ± 0.26
Decomposed SIN-1	8.18 ± 1.32	10.1 ± 1.08	1.12 ± 0.12
+ Cf	17.5 ± 2.65*	30.5 ± 3.56*	6.19 ± 0.65*
+ Cf + Ry	8.80 ± 0.98(*)	10.4 ± 1.17(*)	1.32 ± 0.25(*)
SIN-1 (1 mM)	18.6 ± 2.20*	32.0 ± 3.30*	5.89 ± 0.68*
Decomposed SIN-1	8.67 ± 1.52	11.0 ± 1.50	0.94 ± 0.14
+ Cf	18.9 ± 1.98*	31.0 ± 4.10*	5.94 ± 0.49*
+ Ry	9.12 ± 1.15(*)	11.6 ± 2.23(*)	1.28 ± 0.09(*)
+ Cf + Ry	9.26 ± 1.17(*)	11.8 ± 2.21(*)	1.25 ± 0.31(*)

Cells were exposed for 10 (Rhod 2-fluorescence) or 30 min (MitoSOX red-fluorescence and DNA-strand scission) to 0, 0.1, or 1 mM SIN-1, with or without prior addition of Ry and/or Cf. After treatments, the cells were processed as indicated in Materials and Methods. Results represent the mean ± SD from four separate experiments. *$p < 0.001$ as compared with untreated cells; (*)$p < 0.001$ as compared with cells treated with SIN-1 alone or SIN-1 associated with Cf (ANOVA followed by Dunnett's test).

Table 5. Effects of CF or Antimycin A on Aconitase Activity of U937 Cells Exposed to Low Concentrations of Peroxynitrite

Treatment	*Aconitase activity (mU/mg proteins)*
Untreated	8.17 ± 0.11
Cf	8.09 ± 0.89
Antimycin A	7.98 ± 0.92
Peroxynitrite (40 μM)	7.53 ± 0.2
+Cf	5.56 ± 0.36*
+ Cf + Ry	7.57 ± 0.42(*)
+ Cf + Rotenone	5.14 ± 0.75*
+ Antimycin A	5.44 ± 0.57*
+ Antimycin A + Ry	4.93 ± 0.98*
+ Antimycin A + Rotenone	8.05 ± 0.86(*)

U937 cells were exposed for 10 (peroxynitrite/antimycin A) or 30 (peroxynitrite/Cf) min to 0 or 40 μM peroxynitrite, with or without prior addition of Ry or rotenone and/or Cf or antimycin A. After treatments, the cells were processed as indicated in Materials and Methods. Results represent the mean ± SD from four separate experiments.

*$p < 0.001$ as compared with untreated cells.

(*)$p < 0.001$ as compared with cells treated with peroxynitrite associated with Cf or antimycin A (one-way ANOVA followed by Dunnett's test).

enzyme tightly bound to the matrix side of the inner mitochondrial membrane (20). Studies are in progress to identify the mechanism of superoxide formation under the specific conditions used in this study.

The first information providing evidence for CDM derives from experiments performed in permeabilized cells, in which Ca^{2+} as well as additional poorly membrane-permeant compounds can be directly delivered into the cytosol. We observed that 40 μM peroxynitrite promotes DNA-strand scission in the presence of increasing Ca^{2+} concentrations through mechanisms unaffected by manipulations abolishing DNA cleavage, mediated by either CRDM or RDM, as exposure to rotenone or myxothiazol, or the respiration-deficient phenotype (Fig. 1). It was interesting to note that the low concentration of peroxynitrite nevertheless produces maximal DNA-strand scission, as identical Ca^{2+}-dependent responses are observed by using fivefold greater peroxynitrite concentrations in cells manipulated to prevent CRDM (Figs. 1C and 2). As noted earlier for RDM, it is intriguing that maximal responses for DNA-strand scission are obtained under conditions in which events, likely to occur *in vivo* as mitochondrial Ca^{2+} accumulation, take place concomitant with exposure to levels of peroxynitrite otherwise unable to generate detectable DNA cleavage.

Collectively, these results point to CDM as a novel mechanism for DNA-strand scission induced by low concentrations of peroxynitrite, entirely dependent on mitochondrial Ca^{2+} accumulation and, based on data once again obtained with the permeabilized cell system, mediated by H_2O_2 generated at the mitochondrial level (Table 1), as CRDM (16) or RDM (11).

The Ca^{2+} requirements, at the same peroxynitrite concentrations, were, however, remarkably different for CDM and CRDM. We were able to suppress CDM with Ry, or by using DMSO-differentiated U937 cells/human monocytes, and obtain maximal DNA-strand scission with 200 μM peroxynitrite and 3 μM Ca^{2+} through a mechanism qualifying as CRDM, in that it was sensitive to both rotenone and myxothiazol (Fig. 2). This is about one order of magnitude less Ca^{2+} than the amount necessary to elicit a maximal response through CDM.

Having established the existence of CDM in permeabilized cells, we moved to intact cells to assess the biologic relevance of this mechanism. To promote mitochondrial Ca^{2+} accumulation, we used a strategy based on exposure of U937 cells to agents mobilizing the cation from the RyR (16). We reproduced these results, indicating that Cf promotes this event through an Ry-sensitive mechanism and noted that, under the same conditions, peroxynitrite mediates formation of superoxide/H_2O_2 and the ensuing DNA cleavage after exposure to concentrations of peroxynitrite, failing to promote effects in the absence of Cf (Fig. 3). The notion that the mechanism involved in these responses is independent of electron transport in the respiratory chain was clearly established by using specific inhibitors (Fig. 3) and respiration-deficient cells (Fig. 4). Cf also was found to promote peroxynitrite-dependent inhibition of aconitase activity and, once again, this effect was insensitive to rotenone (Table 5). Conversely, rotenone prevented the enhancing effects mediated by antimycin A, thereby providing an important control, supporting our conclusion that the Cf-dependent responses under investigation are independent of effects on the respiratory chain.

The enhancing effects of Cf are attributable to mobilization of Ca^{2+} from the RyR and subsequent mitochondrial Ca^{2+} accumulation. Superoxide/H_2O_2 formation and DNA-strand scission were indeed found to take place under the same conditions in which mitochondrial Ca^{2+} accumulation was also observed, and both events were blunted by Ry (Fig. 3). In addition, Cf failed to promote effects in cells not expressing the RyR, as the DMSO-differentiated U937 cells or human monocytes (Table 2). Experiments performed in permeabilized cells indicated that Cf mimics the effects of reagent Ca^{2+} in respiration-proficient and -deficient U937 cells, whereas hardly any effect was noticed in cells failing to express the RyR (Table 1). As a final note, 4-CmC, a structurally unrelated agonist of the RyR, reproduced all the effects mediated by Cf (Table 3).

Collectively, results obtained with intact cells confirm the outcome of studies performed in permeabilized cells. It also should be noted that these effects detected in intact cells were not the consequence of nonspecific toxic events, as the conditions used (*i.e.*, peroxynitrite with or without Cf) failed to produce obvious signs of toxicity and even failed to affect the proliferation rates of the cells. Finally, it also was demonstrated that SIN-1, a peroxynitrite donor, mimics the effects obtained with exogenous peroxynitrite (Table 4).

In conclusion, our results provide solid experimental evidence for a novel mechanism, CDM, leading to DNA damage (and inhibition of aconitase activity) under conditions of enforced mitochondrial Ca^{2+} accumulation and exposure to low concentrations of peroxynitrite. Under these conditions, CDM mediates the mitochondrial formation of species producing strand scission of genomic DNA through a mechanism completely independent of effects on electron transport. We also may predict that other agents, or conditions associated with the inflammatory response, leading to Ca^{2+} mobilization from other intracellular pools, or cellular uptake from the extracellular milieu, may promote enhanced DNA-strand

scission, provided that the cation is effectively cleared by the mitochondria. All these hypotheses and possibilities can be experimentally tested.

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Author Disclosure Statement

No competing financial interests exist.

References

1. Beckman JS, Beckman TW, Chen J, Marshall PA, and Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 87: 1620–1624, 1990.
2. Brand K. Aerobic glycolysis by proliferating cells: protection against oxidative stress at the expense of energy yield. *J Bioenerg Biomembr* 29: 355–364, 1997.
3. Cantoni O and Guidarelli A. Indirect mechanisms of DNA strand scission by peroxynitrite. *Methods Enzymol* 440: 111–120, 2008.
4. Carafoli E. Intracellular calcium homeostasis. *Annu Rev Biochem* 56: 395–433, 1987.
5. Douki T and Cadet J. Peroxynitrite mediated oxidation of purine bases of nucleosides and isolated DNA. *Free Radic Res* 24: 369–380, 1996.
6. Douki T, Cadet J, and Ames BN. An adduct between peroxynitrite and 2′-deoxyguanosine: 4,5-dihydro-5-hydroxy-4-(nitrosooxy)-2′-deoxyguanosine. *Chem Res Toxicol* 9: 3–7, 1996.
7. Epe B, Ballmaier D, Roussyn I, Briviba K, and Sies H. DNA damage by peroxynitrite characterized with DNA repair enzymes. *Nucleic Acids Res* 24: 4105–4110, 1996.
8. Feissner RF, Skalska J, Gaum WE, and Sheu SS. Crosstalk signaling between mitochondrial Ca^{2+} and ROS. *Front Biosci* 14: 1197–1218, 2009.
9. Fiskum G, Craig SW, Decker GL, and Lehninger AL. The cytoskeleton of digitonin-treated rat hepatocytes. *Proc Natl Acad Sci USA* 77: 3430–3434, 1980.
10. Gardner PR. Aconitase: sensitive target and measure of superoxide. *Methods Enzymol* 349: 9–23, 2002.
11. Guidarelli A, Cerioni L, and Cantoni O. Inhibition of complex III promotes loss of Ca^{2+} dependence for mitochondrial superoxide formation and permeability transition evoked by peroxynitrite. *J Cell Sci* 120: 1908–1914, 2007.
12. Guidarelli A, Cerioni L, Fiorani M, and Cantoni O. Differentiation-associated loss of ryanodine receptors: a strategy adopted by monocytes/macrophages to prevent the DNA single-strand breakage induced by peroxynitrite. *J Immunol* 183: 4449–4457, 2009.
13. Guidarelli A, De Sanctis R, Cellini B, Fiorani M, Dacha M, and Cantoni O. Intracellular ascorbic acid enhances the DNA single-strand breakage and toxicity induced by peroxynitrite in U937 cells. *Biochem J* 356: 509–513, 2001.
14. Guidarelli A, Fiorani M, and Cantoni O. Enhancing effects of intracellular ascorbic acid on peroxynitrite-induced U937 cell death are mediated by mitochondrial events resulting in enhanced sensitivity to peroxynitrite-dependent inhibition of complex III and formation of hydrogen peroxide. *Biochem J* 378: 959–966, 2004.
15. Guidarelli A, Fiorani M, Tommasini I, Cerioni L, and Cantoni O. Reduced mitochondrial formation of H_2O_2 is responsible for resistance of dimethyl sulfoxide differentiated U937 cells to peroxynitrite. *Int J Biochem Cell Biol* 38: 56–68, 2006.
16. Guidarelli A, Sciorati C, Clementi E, and Cantoni O. Peroxynitrite mobilizes calcium ions from ryanodine-sensitive stores, a process associated with the mitochondrial accumulation of the cation and the enforced formation of species mediating cleavage of genomic DNA. *Free Radic Biol Med* 41: 154–164, 2006.
17. Guidarelli A, Tommasini I, Fiorani M, and Cantoni O. Essential role of the mitochondrial respiratory chain in peroxynitrite-induced strand scission of genomic DNA. *IUBMB Life* 50: 195–201, 2000.
18. Hajnoczky G, Robb-Gaspers LD, Seitz MB, and Thomas AP. Decoding of cytosolic calcium oscillations in the mitochondria. *Cell* 82: 415–424, 1995.
19. Inoue S and Kawanishi S. Oxidative DNA damage induced by simultaneous generation of nitric oxide and superoxide. *FEBS Lett* 371: 86–88, 1995.
20. Maas E and Bisswanger H. Localization of the alpha-oxoacid dehydrogenase multienzyme complexes within the mitochondrion. *FEBS Lett* 277: 189–190, 1990.
21. Massey V. Activation of molecular oxygen by flavins and flavoproteins. *J Biol Chem* 269: 22459–22462, 1994.
22. Mukhopadhyay P, Rajesh M, Hasko G, Hawkins BJ, Madesh M, and Pacher P. Simultaneous detection of apoptosis and mitochondrial superoxide production in live cells by flow cytometry and confocal microscopy. *Nat Protoc* 2: 2295–2301, 2007.
23. Niles JC, Wishnok JS, and Tannenbaum SR. Peroxynitrite-induced oxidation and nitration products of guanine and 8-oxoguanine: structures and mechanisms of product formation. *Nitric Oxide* 14: 109–121, 2006.
24. Pacher P, Beckman JS, and Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 87: 315–424, 2007.
25. Tahara EB, Barros MH, Oliveira GA, Netto LE, and Kowaltowski AJ. Dihydrolipoyl dehydrogenase as a source of reactive oxygen species inhibited by caloric restriction and involved in *Saccharomyces cerevisiae* aging. *FASEB J* 21: 274–283, 2007.
26. Thomas CE and Reed DJ. Effect of extracellular Ca^{++} omission on isolated hepatocytes, II. Loss of mitochondrial membrane potential and protection by inhibitors of uniport Ca^{++} transduction. *J Pharmacol Exp Ther* 245: 501–507, 1988.
27. Tommasini I, Sestili P, and Cantoni O. Delayed formation of hydrogen peroxide mediates the lethal response evoked by peroxynitrite in U937 cells. *Mol Pharmacol* 61: 870–878, 2002.
28. Tommasini I, Sestili P, Guidarelli A, and Cantoni O. Hydrogen peroxide generated at the level of mitochondria in response to peroxynitrite promotes U937 cell death via inhibition of the cytoprotective signalling mediated by cytosolic phospholipase A_2. *Cell Death Differ* 11: 974–984, 2004.
29. Trollinger DR, Cascio WE, and Lemasters JJ. Mitochondrial calcium transients in adult rabbit cardiac myocytes: inhibition by ruthenium red and artifacts caused by lysosomal loading of Ca^{2+}-indicating fluorophores. *Biophys J* 79: 39–50, 2000.
30. Virag L, Scott GS, Antal-Szalmas P, O'Connor M, Ohshima H, and Szabo C. Requirement of intracellular calcium mobilization for peroxynitrite-induced poly(ADP-ribose) synthetase activation and cytotoxicity. *Mol Pharmacol* 56: 824–833, 1999.
31. Whiteman M, Armstrong JS, Cheung NS, Siau JL, Rose P, Schantz JT, Jones DP, and Halliwell B. Peroxynitrite mediates

calcium-dependent mitochondrial dysfunction and cell death via activation of calpains. *FASEB J* 18: 1395–1397, 2004.
32. Yermilov V, Rubio J, Becchi M, Friesen MD, Pignatelli B, and Ohshima H. Formation of 8-nitroguanine by the reaction of guanine with peroxynitrite in vitro. *Carcinogenesis* 16: 2045–2050, 1995.
33. Zorzato F, Scutari E, Tegazzin V, Clementi E, and Treves S. Chlorocresol: an activator of ryanodine receptor-mediated Ca^{2+} release. *Mol Pharmacol* 44: 1192–1201, 1993.

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Abbreviations Used

4-CmC = 4-chloro-*m*-cresol
CDM = Ca^{2+}-dependent/respiratory chain-independent mechanism
Cf = caffeine
CRDM = Ca^{2+}-/respiratory chain-dependent mechanism
DHR = dihydrorhodamine 123
dihydro-Rhod 2-AM = dihydro-Rhod 2-acetoxymethyl ester
DMSO = dimethylsulfoxide
RDM = respiration-dependent/Ca^{2+}-independent mechanism
RR = ruthenium red
Ry = ryanodine
RyR = ryanodine receptor
SIN-1 = 3-morpholinosydnonimine