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Differentiation-Associated Loss of Ryanodine Receptors: A Strategy Adopted by Monocytes/Macrophages to Prevent the DNA Single-Strand Breakage Induced by Peroxynitrite

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Monocytes/macrophages respond to peroxynitrite with the triggering of events leading to prevention of an otherwise prompt lethal response. This survival signaling regulated by molecules of the arachidonate cascade however presents a hypothetical critical limitation. In human promonocytic cell lines, peroxynitrite indeed promotes ryanodine receptor-derived Ca²⁺-dependent mitochondrial formation of H₂O₂, entirely responsible for the ensuing DNA strand scission. The occurrence of the same events in monocytes/macrophages at the inflammatory sites would therefore enhance the extent of DNA strand scission in viable cells, thereby increasing the rate of mutation and neoplastic transformation. The present study illustrates the details of a novel strategy based on a differentiation-associated loss of expression of ryanodine receptors. These cells simply do not accumulate mitochondrial Ca²⁺ in response to peroxynitrite and therefore fail to generate superoxide/H₂O₂, thereby preserving the integrity of their DNA. We propose that an important component of the overall strategy adopted by monocytes/macrophages to survive to peroxynitrite, with no increased risk of neoplastic transformation, involves down-regulation of ryanodine receptor expression. The Journal of Immunology, 2009, 183: 4449–4457.

Peroxynitrite, the coupling product of superoxide and NO, is produced by macrophages and other inflammatory cells in response to a variety of proinflammatory stimuli. This species promotes both direct and indirect damage in different biomolecules of target cells (1), including the DNA, and an extensive literature documents its involvement in a variety of chronic inflammatory diseases (1, 2) as well as in carcinogenesis (1). Much less is known on the effects of peroxynitrite in peroxynitrite-producing cells, in particular cells participating in the inflammatory response. These cells are obviously affected by their own peroxynitrite and must be able to cope with these lesions to survive and perform energy-demanding functions under inflammatory conditions.

In recent years, our laboratory has been actively involved in studies focusing on the effects of peroxynitrite in cells belonging to the monocyte/macrophage lineage. We showed that the resistance phenotype is dependent on the ability of these cells to respond to peroxynitrite with the triggering of a survival signaling in which cytosolic phospholipase A₂, 5-lipoxygenase, and protein kinase C are sequentially involved (3–6), leading to Bad phosphorylation and to its cytosolic accumulation (5–7). Loss of mitochondrial Bad creates optimal conditions for the antimitochondrial permeability transition function of Bcl-2/Bcl-x₅ (8). Hence, preventing each of the above events was invariably associated with the mitochondrial translocation of Bad and Bax and with an ensuing mitochondrial permeability transition-dependent death. Interestingly, under these conditions, cells can still be rescued by a signaling triggered by prostaglandins that, via PGE₂ receptor-mediated protein kinase A activation, also promote Bad phosphorylation (9). Thus, although the Akt-dependent pathway leading to Bad phosphorylation is severely inhibited by peroxynitrite (9), different species largely available at the inflammatory sites converge in Bad inactivation and promote survival.

The above results identify an ingenious survival strategy based on the ability of monocytes/macrophages to respond to signaling molecules largely available at the inflammatory sites, i.e., under the same conditions in which peroxynitrite is being formed. There is however a potential caveat with this survival strategy, as peroxynitrite is a potent DNA-damaging species (1). The rate of mutation and neoplastic transformation should be indeed significantly enhanced under conditions in which monocytes/macrophages preserve their viability but nevertheless accumulate extensive lesions in their DNA.

The susceptibility of monocytes/macrophages to DNA cleavage induced by peroxynitrite has not been yet investigated in our laboratory, in which however pioneer studies unraveled the mechanism of DNA strand scission induced by the oxidant in U937 cells (a promonocytic cell line). Somewhat unexpectedly, we found that the very large majority of the DNA breaks is not directly induced by peroxynitrite but is rather mediated by secondary H₂O₂ formation (10). We also reported that H₂O₂ is rapidly produced at the mitochondrial level upon dismutation of superoxide, generated via a mechanism dependent on both inhibition of electron transport at the complex III level (10, 11) and on mitochondrial Ca²⁺ accumulation (11, 12). In addition, the fraction of the cation accumulating in the mitochondrial compartment was found to derive from the ryanodine (Ry)⁴ receptor (RyR) (12).

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4 Abbreviations used in this paper: Ry, ryanodine; 4-CmC, 4-chloro-m-cresol; DHR, dihydrorhodamine 123; U937, differentiated U937 cell; U-U937, undifferentiated U937 cell; RR, ruthenium red; RyR, Ry receptor; Cf, caffeine.

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Materials and Methods

Chemicals

Antimycin A, H2O2, rotenone, myxothiazol, Ry, 4-chloro-n-cresol (4-CmC), caffeine (Cf), ruthenium red (RR), LaCl3, CaCl2, catalase, and DMSO as well as most reagent grade chemicals were obtained from Sigma-Aldrich. TGF-B was from PeproTech. DHR and Rhod 2-aceoxymethyl ester were from Molecular Probes.

Cell culture and treatments

U937 human myeloid leukemia cells were cultured in suspension in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS (Euroclone; Celbio Biotecnologie), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Euroclone) at 37°C in T-75 tissue culture flasks (Corning) gassed with an atmosphere of 95% air-5% CO2.

These cells (U937) were differentiated to monocytes (D-U937) by a 4-day growth in culture medium supplemented with 1.3% DMSO as previously described (13).

Human peripheral mononuclear cells were obtained fromuffy coats of healthy blood donors through the courtesy of Centro Trasfusionale, Ospedale civile “Fraternitá 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 1640 medium. Nonadherent cells were removed by repeated washing with PBS (8 g/L NaCl, 1.15 g/L NaHPO4, 0.2 g/L KH2PO4 and 0.2 g/L KCl) while adherent cells were subsequently scraped with trypsin and then used for monocyte experiments. Macrophages were obtained by culturing monocytes (1 x 107 cells/ml) for 8-10 days in RPMI 1640 medium. Macrophages were scraped with trypsin, centrifuged, and used for experiments.

In some experiments, D-U937 or monocytes were incubated for 24 h with 1 ng/ml TGF-B in RPMI 1640 medium before experimentation.

Experiments with intact cells were performed using 15- or 50-ml plastic tubes containing 2.5 x 107 cells/ml prewarmed saline A (8.182 g/L NaCl, 0.372 g/L KCl, 0.336 g/L NaHCO3, and 0.9 g/L glucose). Similar conditions were used in experiments employing permeabilized cells. Permeabilization was achieved by adding digitonin (10 µM) to a medium consisting of 0.25 M sucrose, 0.1% (w/v) BSA, 10 mM MgCl2, 10 mM K HEPES, and 5 mM KH2PO4 (pH 7.2) at 37°C. Under these conditions, digitonin permeabilizes the plasma membrane but leaves mitochondrial membranes intact (14).

Peroxynitrite, synthesized as previously described (15), was rapidly added on the wall of the plastic tubes and mixed for a few seconds 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 1.5 N HCl was also added.

Stock solutions of RR, Cf, CaCl2, LaCl3, and catalase were freshly prepared in distilled water. Ry, antimycin A, myxothiazol, and 4-CmC were dissolved in 95% (v/v) ethanol. Rotenone was 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 cytochrome oxidase properties of peroxynitrite or H2O2.

Measurement of mitochondrial Ca2+

Cells were first exposed for 30 min (4°C) to 10 µM Rhod 2-aceoxymethyl ester, washed three 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 loading of Rhod 2 into the mitochondria (16). The cells were washed three times and treated for 10 min with Cf, 4-CmC, peroxynitrite, or peroxynitrite/Cf in the absence or presence of Ry in 35-mm tissue culture dishes containing an uncoated coverslip. After treatments, the cells were washed three times and fluorescence images were captured with a BX-51 microscope (Olympus) equipped with a SPOT-RT camera unit (Diagnostic Instruments) using an Olympus LC/ACH x40/0.55 objective lens.

The excitation and emission wavelengths were 540 and 590 nm, respectively, with a 55-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 using Scion Image software. Mean fluorescence values were determined by averaging the fluorescence values of at least 50 cells per treatment condition per experiment.

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

DNA single-strand breakage was determined using the alkaline halo assay developed in our laboratory (11). Details on the processing of fluorescence images and on the calculation of the experimental results are also given in (11). DNA single-strand breakage 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 plus halo) and that of the nucleus, from 50 to 75 randomly selected cells per experiment per 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 oxidation and imaging

Cells were first exposed for 3 min to peroxynitrite in 35-mm tissue culture dishes containing an uncoated coverslip and were postincubated for a further 27 min in the presence of 10 µM DHR. After treatments, the cells were washed three times and analyzed with a fluorescence microscope. The resulting images were taken and processed as described above. The excitation and emission wavelengths were 488 and 515 nm, respectively, with a 5-nm slit width for both emission and excitation. Mean fluorescence values were determined by averaging the fluorescence values of at least 50 cells per treatment condition per experiment.

Immunocytochemical detection of nitrotyrosine

Cells were incubated for 10 min in 2 ml of saline A in 35-mm tissue culture dishes containing an uncoated coverglass and were postincubated for a further 27 min in the presence of 10 µM DHR. After treatments, the cells were fixed for 1 min with 95% ethanol/5% acetic acid, washed with PBS, and blocked in PBS containing BSA (2%, w/v) for 30 min at room temperature. Thereafter, the cells were incubated with rabbit polyclonal anti-nitrotyrosine Ab (5 µg/ml in PBS containing 1% BSA). After 18 h at 4°C, the cells were washed and subsequently exposed to FITC-conjugated secondary Ab diluted 1/200 in PBS. After a 2-h incubation in the dark, stained cells were analyzed using a fluorescence microscope and the resulting images were processed for fluorescence determination as described above.

Western blot analysis

U-D-U937 cells as well as human monocytes and macrophages were lysed as described previously (7). Male Sprague Dawley rats weighing 250–300 g were anesthetized with sodium pentobarbital and killed by decapitation. All surgical and experimental procedures were conducted in accordance with the Italian regulation for the care and use of laboratory animals and were approved by appropriate institutional and state authorities. The hearts were then rapidly removed, rinsed in ice-cold PBS, and disrupted with a Dounce homogenizer in a buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 25 µg/ml p-nitrophe- nylglycinephenylalanilinate. Muscle homogenates were then incubated for 20 min at 4°C. After centrifugation at 14,000 x g for 15 min, supernatants were collected and analyzed for total protein. Western blot analysis was next performed using a RyR (obtained from Affinity Bioreagents) Ab recognizing both the type 1 and type 2 RyR isoforms. Details on Western blotting apparatus and conditions are reported elsewhere (17).
Nonprotein thiol assay

Cellular nonprotein thiol content was determined as described elsewhere (18). In brief, cells (4 × 10^6) were washed three times with saline A, centrifuged, and the pellet was subsequently resuspended in 150 μl of a metaphosphoric acid solution (1.67% (v/v) metaphosphoric acid/0.2% EDTA/30% (w/v) NaCl). After a 5-min incubation in an ice bath, the cell suspension was centrifuged for 5 min at 10,000 × g. The nonprotein thiol content was measured spectrophotometrically in the supernatant, at 412 nm using 5,5'-dithiobis(2-nitrobenzoic acid) (ε_{412} = 13,600 M⁻¹cm⁻¹).

Catalase activity

Catalase activity was determined spectrophotometrically in the supernatant as previously described (18). Briefly, the cells were washed twice with saline A, resuspended in the same medium at a density of 5 × 10^6 cells/ml, and finally sonicated (15 s) three times on ice with a Branson sonifier operating at 20 W. The resulting homogenates were centrifuged for 5 min at 18,000 × g at 4°C.

Statistical analysis

The results are expressed as means ± 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

Monocytes are resistant to the DNA cleavage induced by peroxynitrite

Increasing concentrations of peroxynitrite cause oxidation of DHR (Fig. 1A) and DNA single-strand breakage (Fig. 1B) in U-U937 and D-U937, but not in monocytes. These results indicate that monocytes are resistant to peroxynitrite-dependent H₂O₂ formation and to the ensuing DNA cleavage: the resistance phenotype is circumvented by antimycin A.
cells, with a maximal response at 200 μM. Under the same conditions, however, monocytes were resistant to the effects of peroxynitrite, barely detectable only at the highest concentration tested. Lack of sensitivity to the DNA-damaging effects of peroxynitrite was reproduced in monocytes from 35 healthy individuals (data not shown). The same resistance phenotype is acquired by U937 cells upon differentiation to monocytes following prolonged exposure to DMSO (D-U937 cells).

These results indicate that monocytes are resistant to DHR oxidation that under the above conditions reflect formation of mitochondrial H2O2 (19) and thus to the DNA-damaging effects of peroxynitrite, almost entirely mediated by H2O2 (10, 12).

Resistance is not associated with changes in the peroxynitrite/H2O2-scavenging capacity

The results illustrated in Table I indicate that 100 μM peroxynitrite promotes comparable levels of nitrotyrosine immunoreactivity in monocytes and in U-/D-U937 cells. In addition, the GSH pool is mobilized or to a differentiation-associated loss of functional RyRs. Peroxynitrite-dependent mitochondrial formation of superoxide/H2O2 in the absence of bona fide complex III inhibitors (12).

Reduced mitochondrial formation of H2O2 is associated with impaired mitochondrial Ca2+ accumulation

As shown in Fig. 2A, peroxynitrite (100–200 μM) causes in U937 cells a Rhod 2-fluorescence response sensitive to Ry (20 μM). This response was dependent on enhanced mitochondrial Ca2+ accumulation and, indeed, as we recently reported (12, 20), Rhod 2-fluorescence colocalized with the fluorescence of a mitochondrial probe (data not shown). Under identical treatment conditions, however, there was no evidence of Rhod 2-derived fluorescence in D-U937 cells or monocytes.

These results suggest that resistance of D-U937 cells and monocytes to secondary H2O2 formation and DNA strand scission mediated by peroxynitrite is attributable to the lack of mitochondrial Ca2+ accumulation. The results illustrated in Fig. 2, E and F, are consistent with this notion as they indicate loss of the resistance phenotype under conditions of enforced mitochondrial Ca2+ accumulation. Identical levels of DNA strand scission were indeed detected in permeabilized U- and D-U937 cells, or monocytes, supplemented with peroxynitrite (40 μM) and increasing concentrations of Ca2+ (Fig. 2E). Ca2+ did not produce effects in the absence of peroxynitrite and the oxidant produced very low levels of breaks in the absence of Ca2+. In addition (Fig. 2F), the DNA damage mediated by peroxynitrite and Ca2+ in the three different cell types was sensitive to rotenone and enzymatically active catalase, as expected, as well as to 200 nM RR (that under these conditions specifically prevents mitochondrial Ca2+ uptake) (22) and lanthanum ions (100 μM, known to competitively inhibit Ca2+ uptake) (23).

Collectively, the above results suggest that D-U937 cells and monocytes are resistant to DNA strand scission mediated by peroxynitrite because they fail to accumulate mitochondrial Ca2+, presumably as a consequence of a differentiation-associated loss of functional RyRs. Peroxynitrite-dependent mitochondrial formation of H2O2, largely responsible for DNA single-strand breakage (10) is consequently prevented.

Resistance is due to loss of expression of RyRs

The notion that resistance of D-U937 cells and monocytes to secondary H2O2 formation/DNA strand scission mediated by peroxynitrite is attributable to the lack of mitochondrial Ca2+ accumulation may be due to either resistance of the RyR to peroxynitrite-dependent Ca2+ mobilization or to a differentiation-associated loss of functional RyRs.

High concentrations of CF (10 mM) promote the efflux of Ca2+ from Ry-sensitive stores in an array of different cell types (24). As we previously showed (12), the initial increase in cytosolic Ca2+ mediated by CF in U-U937 cells is associated with a Ry-sensitive increase in the mitochondrial fraction of the cation (Fig. 2B). Once
again, there was no evidence of Rhod 2 fluorescence in D-U937 cells or monocytes exposed to Cf. Similar results were obtained with 4-CmC (500 μM), an agent also promoting the efflux of Ca²⁺ from Ry-sensitive stores (25). Measurements of H₂O₂ formation (Fig. 2C) and DNA strand scission (Fig. 2D) in U-U937 cells reproduced the previously established ability of Ry to suppress both events maximally induced by 200 μM peroxynitrite (12). In addition, Cf or 4-CmC (data not shown) promoted maximal effects via a Ry-sensitive mechanism after exposure to very low concentrations of peroxynitrite (e.g., 40 μM). Treatment with the sole Cf, or
4-CmC, did not cause DHR oxidation or DNA single-strand breakage (data not shown).

Consistent with these findings, Cf (or 4-CmC; data not shown) failed to elicit formation of \( \text{H}_2\text{O}_2 \) and DNA strand scission in D-U937 cells or monocytes exposed to 40 or 200 \( \mu \text{M} \) peroxynitrite. The above findings are therefore consistent with the notion that D-U937 cells and monocytes do not express a functional RyR.

The results illustrated in Fig. 3 indicate U-U937 cell immunoreactivity to Ab recognizing the RyR, comigrating with a similar band from rat cardiac muscle lysates. This band is not observed in

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**FIGURE 3.** Differentiation-associated loss of RyRs is responsible for resistance of monocytes or macrophages to peroxynitrite-dependent \( \text{H}_2\text{O}_2 \) formation and DNA cleavage. A, Cardiac muscle homogenates (20 \( \mu \text{g} \) of protein) as well as lysates (75 \( \mu \text{g} \) of protein) from U-U937, D-U937, monocytes, or macrophages were processed for Western blot analysis using an anti-RyR Ab. The relative amount of RyR protein was quantified by densitometric analysis. Lack of RyR expression was consistently observed in monocytes and macrophages from 10 and 6 separate individuals, respectively. B, U-U937 grown for 0–96 h in the presence of DMSO were processed as indicated in A. The relative amount of RyR protein was quantified by densitometric analysis. Not detectable. C–E, U-U937 cells were grown for increasing time intervals in the presence of DMSO and then incubated for 10 (C) or 30 (D and E) min with 0 (○) or 200 \( \mu \text{M} \) (●) peroxynitrite. After treatments, the cells were analyzed for Rhod 2 fluorescence (C), DHR fluorescence (D), and DNA damage (E) as detailed in Materials and Methods. F, Rhod 2-acetoxymethyl ester-preloaded macrophages were exposed for 10 min to the vehicle, Cf, or 200 \( \mu \text{M} \) peroxynitrite before fluorescence microscopy analysis. G and H, Macrophages were first treated for 5 min with the vehicle, Cf, rotenone, or myxothiazol, subsequently exposed for 3 min to 200 \( \mu \text{M} \) peroxynitrite, and finally incubated for 27 min with either the vehicle or antimycin A. After treatments, cells were analyzed for DHR fluorescence (G) and DNA damage (H) as detailed in Materials and Methods. Results represent the means ± SD calculated from three to five separate experiments. #, \( p < 0.01 \); ##, \( p < 0.001 \) compared with untreated cells (two-way ANOVA followed by Bonferroni’s test). * \( p < 0.001 \) compared with untreated cells (one-way ANOVA followed by Dunnett’s test). Blots shown are representative of three separate experiments with similar outcomes.
D-U937 cells, monocytes (replicated in cells from 10 individuals), or human macrophages (replicated in cells from six individuals). Interestingly, growth of U937 cells in the presence of DMSO promotes time-dependent loss of immunoreactivity (Fig. 3B), an event paralleled by loss of mitochondrial Ca\(^{2+}\) accumulation (Fig. 3C), delayed H\(_2\)O\(_2\) formation (Fig. 3D), and DNA strand scission (Fig. 3E). It should be noted that, as previously observed in monocytes, macrophages fail to accumulate mitochondrial Ca\(^{2+}\) in response to peroxynitrite or Cf (Fig. 3F). In addition, macrophages were also resistant to DHR oxidation (Fig. 3G) or DNA strand scission (Fig. 3H) induced by peroxynitrite alone or associated with Cf. Finally, antimycin A promoted maximal H\(_2\)O\(_2\) formation and DNA cleavage also in macrophages, once again via a rotenone- or myxothiazol-sensitive mechanism.

There are reports indicating that Jurkat T cells, unlike U937 cells, only express detectable levels of RyR isoforms after stimulation with specific cytokines, such as TGF-\(\beta\) (26). We therefore tested whether treatment with TGF-\(\beta\) (24 h) also causes RyR expression in D-U937 cells or monocytes and obtained a positive response in terms of both Western blot analysis (Fig. 4A) and detection of Ry-sensitive Rhod 2 fluorescence in response to either Cf or 200 \(\mu\)M peroxynitrite (Fig. 4B). Under the same conditions, Cf promoted Ry-sensitive formation of H\(_2\)O\(_2\) (Fig. 4C) and DNA strand scission (Fig. 4D) after exposure to 40 \(\mu\)M peroxynitrite. Along the same lines, pretreatment with TGF-\(\beta\) also leads to the reappearance of Ry-sensitive secondary H\(_2\)O\(_2\) formation (Fig. 4C) and DNA cleavage (Fig. 4D) in response to 200 \(\mu\)M peroxynitrite. The extent of the effects observed in prestimulated D-U937 cells and monocytes was virtually identical to that previously described in U-U937 cells (Fig. 2). Hence, the resistance phenotype of D-U937 cells and monocytes can be circumvented via enforced expression of the RyR.

Collectively, these findings indicate that resistance of monocytes or macrophages to peroxynitrite-dependent H\(_2\)O\(_2\) formation and DNA cleavage is due to a differentiation-associated loss of expression of RyRs.

**Discussion**

Inflammatory cells, as monocytes and macrophages, respond to proinflammatory stimuli with the release of an array of reactive and toxic molecules, including peroxynitrite. Monocytes and macrophages are therefore a primary target of these species but nevertheless survive and perform energy-demanding functions, even under harsh inflammatory conditions. Our work has shown that monocyte/macrophage resistance to peroxynitrite is due to the ability of these cells to respond to arachidonate metabolites with the triggering of a simple, yet very effective survival signaling. Products of either 5-lipoxygenase (5–7) or cyclooxygenase (9), extensively released at the inflammatory sites, initiate a cascade of events culminating in Bad phosphorylation and thus in its cytosolic accumulation. In addition, under these conditions, Bax is also retained in the cytosol and the cells preserve their ATP pool (27), thereby surviving regardless of the damage accumulated (3–7, 9). In particular, our work performed in U937 cells provided evidence of survival of cells with extensive DNA strand scission (27, 28). The occurrence of the same events in monocytes/macrophages at the inflammatory sites would therefore be associated with a potentially increased rate of mutation and neoplastic transformation.

The present study demonstrates that monocytes/macrophages as well as D-U937 cells do not respond to peroxynitrite (as U-U937 cells and monocytes are).
cells) with the mitochondrial formation of H$_2$O$_2$ and are therefore resistant to the DNA-damaging effects of peroxynitrite (Fig. 1). As it will be discussed below, the resistance phenotype is entirely dependent on down-regulation of RyR expression, with hardly any contribution of effects at the level of peroxynitrite/H$_2$O$_2$ scavenging and/or detoxification (Fig. 1 and Table I). This study therefore documents an important difference between a tumor promonocytic cell line (i.e., U-U937 cells) and primary monocytes/macrophages, the first one thus far detected in the complex network regulating survival to peroxynitrite.

Our previous work performed in U-U937 cells showed that secondary H$_2$O$_2$ formation ensues in response to peroxynitrite as a consequence of events involving inhibition of complex III and enforced mitochondrial Ca$^{2+}$ accumulation (12). The results obtained in this study indicate that the resistance phenotype is not associated with resistance to complex III inhibition, as identical levels of rotenone/myxothiazol-sensitive H$_2$O$_2$ formation/DNA strand scission were mediated by peroxynitrite in U-/D-U937 cells, monocytes, and macrophages supplemented with antimycin A (Fig. 1). Since antimycin A promotes maximal H$_2$O$_2$ formation via a Ca$^{2+}$-independent mechanism (20), we conclude that monocytes/macrophages are sensitive to the mitochondrial effects of peroxynitrite.

The resistance phenotype is therefore best explained by lack of mitochondrial Ca$^{2+}$ accumulation, a notion consistent with the observation that D-U937 cells and monocytes gain susceptibility to DNA strand scission induced by peroxynitrite after permeabilization and supplementation of exogenous Ca$^{2+}$ (Fig. 2E). Under these conditions, the DNA single-strand breakage accumulated by the latter cell types was identical to that detected in U-U937 cells. The mechanism involved in the formation of DNA-damaging species was also identical in that it was sensitive to rotenone, myxothiazol, and catalase (Fig. 2F). Hence, the upstream involvement of superoxide, generated in a reaction in which ubiquinone serves as an electron donor and the subsequent formation of H$_2$O$_2$, can be inferred also under these conditions.

Lack of mitochondrial Ca$^{2+}$ accumulation was established in D-U937 cells and monocytes exposed to concentrations of peroxynitrite promoting a Ry-sensitive Rhod 2-fluorescence response in U-U937 cells (Fig. 2A). An identical outcome was obtained in studies using agents that directly mobilize Ca$^{2+}$ from the RyR, namely, Cf or 4-Cmc (Fig. 2B) that also failed to promote peroxynitrite-dependent DHR fluorescence and DNA strand scission (Fig. 2, C and D). These results are in obvious contrast with those obtained in U-U937 cells in which Cf, or 4-Cmc, elicited these effects via a Ry-sensitive mechanism after addition of an otherwise ineffective concentration of peroxynitrite.

All together, these findings strongly suggest that lack of mitochondrial Ca$^{2+}$ accumulation is attributable to lack of expression of RyRs. This notion was clearly established by Western blot studies using Ab against the RyR. There was a positive response in monocytes/macrophages using Ab against the RyR. There was a positive response in monocytes/macrophages, and/or detoxification (Fig. 1 and Table I). This study therefore documents an important difference between a tumor promonocytic cell line (i.e., U-U937 cells) and primary monocytes/macrophages, the first one thus far detected in the complex network regulating survival to peroxynitrite.

Three main types of RyR have thus far been described and named according to the tissue in which they were first identified because of their relative abundance, i.e., the skeletal (RyR1), cardiac (RyR2), and brain (RyR3) isoforms (29, 30). Although all three isoforms appear to be expressed in U-U937 cells (26), the present study does not allow us to conclude on the relevance of each of these specific isoforms in Ca$^{2+}$ mobilization in response to peroxynitrite in either U-U937 cells or in TGF-β-pretreated D-U937 cells and primary monocytes. Future studies will be performed in this direction.

The above results nevertheless unravel an important strategy, based on the loss of expression of RyRs, adopted by monocytes to cope with the DNA-damaging effects of peroxynitrite. This strategy is most likely shared by macrophages also failing to express the RyR (Fig. 3A) and to respond to peroxynitrite with mitochondrial Ca$^{2+}$ accumulation (Fig. 3F), secondary H$_2$O$_2$ formation (Fig. 3G), and DNA strand scission (Fig. 3H).

In conclusion, the results presented in this study indicate that the strategy adopted by monocytes/macrophages to cope with the DNA-damaging effects of peroxynitrite involves down-regulation of RyRs. The comparison with results obtained with monotypic U937 cells, expressing functional RyRs, provides evidence for an obvious advantage. U937 cells survive to the insult mediated by peroxynitrite using the arachidonate-dependent survival signaling (3–7, 9) but nevertheless accumulate extensive DNA damage (27, 28). Monocytes/macrophages use the same pathway to survive to cope with peroxynitrite (5, 9, 31) and are virtually invulnerable to its genotoxic effects. We speculate that down-regulation of RyR expression, responsible for poor mitochondrial Ca$^{2+}$ accumulation in response to peroxynitrite, represents a “convenient” mechanism to ensure survival even under harsh inflammatory conditions with no increased risk of mutation/neoplastic transformation.

Disclosures
The authors have no financial conflict of interest.

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