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Peroxynitrite is a major trigger of cardiomyocyte apoptosis in vitro and in vivo

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Abstract

Recent evidence indicates that peroxynitrite represents a major cytotoxic effector in heart diseases, but its mechanisms of action are still not known exactly. Notably, the ability of peroxynitrite to trigger cardiomyocyte apoptosis, a crucial mode of cell death in many cardiac conditions, remains poorly defined. We evaluated apoptotic and necrotic cell death in cultured H9C2 cardiomyocytes, following a brief (20 min) exposure to peroxynitrite (50–500 μM). Peroxynitrite-dependent myocardial toxicity was then investigated in a rat model of myocardial ischemia-reperfusion (MIR), where the effects of peroxynitrite were blocked by the superoxide dismutase mimetics and peroxynitrite scavenger Mn (III)-tetrakis(4-benzoic acid) porphyrin (MnTBAP). In vitro, peroxynitrite killed cardiomyocytes mostly through apoptosis (DNA fragmentation, apoptotic nuclear alterations, caspase-3 activation, and PARP cleavage), but not necrosis (propidium iodide staining and LDH release). In vivo, MIR triggered myocardial oxidative stress (malondialdehyde generation), nitrotyrosine formation, neutrophil accumulation, and the cleavage of caspase-3 and PARP, indicating ongoing myocardial apoptosis. MnTBAP suppressed these alterations, allowing a considerable reduction of myocardial injury. Thus, peroxynitrite triggers apoptosis in cardiomyocytes in vitro and in the myocardium in vivo, through a pathway involving caspase-3 activation and the cleavage of PARP. These results provide important novel information on the mechanisms of myocardial toxicity of peroxynitrite.

Keywords

Peroxynitrite; Apoptosis; Necrosis; Poly(ADP-ribose) polymerase; Caspase; Myocardium; Cardiomyocyte

Introduction

Peroxynitrite ($ONOO^-$) is a strong biological oxidant and nitrating species formed from the near-diffusion-limited reaction of the free radicals nitric oxide and superoxide anion [1]. $ONOO^-$ formation represents a major mechanism of myocardial injury in various cardiac pathologies including myocardial infarction, chronic heart failure, and cardiomyopathy associated with anthracyclines, diabetes, septic, and hemorrhagic shock (see [2,3] for recent reviews). The myocardial cytotoxicity of $ONOO^-$ involves direct oxidative damage to lipids, proteins, and DNA [3], the activation of metalloproteinases [4], and the nitration of tyrosine residues within proteins [3,5]. We also provided evidence that $ONOO^-$ acts as a potent signaling molecule in cardiomyocytes, activating all members of the MAP kinase family [6],

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and inhibiting the activation of the transcription factor nuclear factor kappa B [7]. Furthermore, a major pathway of $ONOO^-$ dependent myocardial cytotoxicity relies on oxidative DNA damage and activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) [8], which consumes cellular nicotinamide dinucleotide (NAD) and adenosine triphosphate (ATP), leading to cell necrosis [8,9]. Indeed, the prevention of PARP activation markedly reduces cell necrosis in many different cell systems in vitro [10] as well as in various animal models of diseases in vivo, notably myocardial infarction [11,12].

In contrast to its role in cell necrosis, the role of $ONOO^-$ in triggering cardiomyocyte apoptosis has been poorly investigated. Apoptosis is orchestrated by the proteolytic activation of cysteine proteases known as caspases and regulated by proteins belonging to the bcl-2 family. Distinct pathways of apoptosis converge to the activation of executioner caspase-3, which cleaves multiple downstream cellular targets [13]. An important target of caspase-3 is PARP, whose cleavage and inactivation maintains the cellular ATP stores indispensable for apoptosis to proceed properly [14]. Recent evidence has emerged that apoptosis is an important mechanism of cardiomyocyte cell death in conditions such as myocardial reperfusion, cardiomyopathy, and heart failure [15,16]. Such conditions are also associated with enhanced $ONOO^-$ generation and myocardial oxidative stress, which may represent an important mechanism in the development of myocardial apoptosis [16]. So far, only one in vitro study reported that exposure of cardiomyocytes to $ONOO^-$ resulted in apoptotic cell death, via an increased expression of the proapoptotic protein Bax [17], and there is no information regarding the potential proapoptotic role of $ONOO^-$ in vivo. The present study addressed these issues, using both in vitro and in vivo approaches. The mechanisms of $ONOO^-$ mediated cytotoxicity were first determined in cultured rat cardiomyocytes (H9C2 cells) exposed to authentic $ONOO^-$. Secondly, these mechanisms were assessed in an in vivo model of rat myocardial infarction, where the effects of $ONOO^-$ were inhibited by Mn(III)-tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP), a stable and cell-permeable superoxide dismutase mimetic and $ONOO^-$ scavenger [18,19]. Our data show that $ONOO^-$ exerts potent proapoptotic effects in cardiomyocytes in vitro and in the myocardium in vivo, characterized by the activation of caspase-3 and the cleavage of PARP. Thus, $ONOO^-$ may represent a major effector of cardiomyocyte apoptosis contributing to myocardial damage and dysfunction in a wide range of cardiac pathologies.

Materials and methods

In vitro experiments

Cell culture conditions and stimulation with $ONOO^-$—H9C2 cells, a clonal line derived from rat heart (ATCC, Manassas, VA), were grown (5% CO2, 37°C) in DMEM (Gibco BRL, Invitrogen, Basel, Switzerland) supplemented with 10% FBS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. H9C2 cells are morphologically similar to embryonic cardiocytes and are recognized as a well-suited model for the study of cardiomyocyte biology [20]. In addition, we recently reported that $ONOO^-$ activated similar stress signaling responses in H9C2 cells and primary murine cardiomyocytes [6]. $ONOO^-$ (obtained from Calbiochem) was synthesized from isoamyl nitrite and hydrogen peroxide followed by removal of hydrogen peroxide over a manganese dioxide column, as described [21], and was stored in 0.4 M NaOH at −80°C. Prior to experimentation, the concentration of $ONOO^-$ was determined from its absorption at 302 nm (extinction coefficient=1670 M^{-1} cm^{-1}). Stimulation with $ONOO^-$ was made in phosphate-buffered saline to avoid reactions of $ONOO^-$ with media constituents [6, 7]. $ONOO^-$ was delivered as a single bolus at a 1:100 dilution, at final concentrations of 50–500 μM, in agreement with previous studies from our laboratory [6,7] and others [22,23]. Cells were exposed to authentic $ONOO^-$ or decomposed $ONOO^-$ (DP, control condition) for 20 min,

as previously described [6,7], after which they were replaced in complete culture medium for the indicated times.

Cytotoxicity assays—Cell viability was assessed by measuring the mitochondrial-dependent reduction of MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan, as described [24]. The amount of formazan formed was quantified by measuring the absorbance of the solution at 540 nm.

The release of LDH in the culture medium was determined as an indicator of cell necrosis [25,26], using a commercially available kit (Roche Molecular Biochemicals, Basel, Switzerland), as per manufacturer's protocol.

Nuclear morphology by fluorescence microscopy—The fluorescent nuclear binding dyes Hoechst 33342 (Calbiochem), which readily enters cells with intact or damaged membranes and stains DNA blue, and propidium iodide (PI, Sigma Chemicals), which is impermeable to cells with preserved membranes and stains DNA red, were used to detect necrotic and apoptotic cells. Both dyes were added (10 μg/ml, 20 min) to the cells 6 h after a 20-min exposure to $ONOO^-$. Cells were then fixed in 4% paraformaldehyde and nuclear morphology was visualized under a Zeiss fluorescence microscope. Apoptotic cells were detected by typical nuclear morphological changes (chromatin condensation and nuclear fragmentation), whereas necrotic cells were detected by intact nuclei and positive PI staining. In addition, cells at a late stage of apoptosis, where the cell membrane integrity is lost, were detected by typical apoptotic nuclei together with positive PI staining ("secondary" necrosis) [27,28].

Evaluation of apoptosis—Apoptosis was evaluated as follows.

- **a.** Quantification of DNA fragmentation: The Cell Death Detection ELISA kit (Roche Molecular Biochemicals, Basel, Switzerland) was employed to quantify DNA fragmentation on the basis of antibody detection of free histone and fragmented DNA, according to the manufacturer's instruction and previously published protocols [29];
- **b.** Nuclear morphology (see above);
- **c.** Immunoblot detection of cleaved caspase-3 and cleaved PARP.

After stimulation, cells were scraped in lysis buffer (TrisHCl 10 mM, NP40 0.5%, NaCl 0.15 M, Na_3VO_4 1 mM, NaF 10 mM, PMSF 1 mM, EDTA 1 mM, aprotinin 10 μg/ml, leupeptin 10 μg/ml, and pepstatin 1 μg/ml). Proteins (20 μg) were separated by SDS-PAGE, transferred to nitrocellulose membrane, and blocked for 1 h at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20. The membrane was incubated overnight at 4°C with an appropriate dilution of anti-caspase-3 (full-length, 33 kDa), anti-cleaved caspase-3 (17- to19-kDa fragments), anti-PARP-1 (119 kDa), and cleaved-PARP-1 (89 kDa) (all from Cell Signaling, Beverly, MA) primary antibodies, followed by incubation for 1 h with a 1:5000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA).

In vivo experiments

Surgical procedure—All procedures were in accordance with the Swiss laws on animal experimentation and conformed with the principles outlined in the Declaration of Helsinki. Myocardial ischemia-reperfusion was induced as described previously [11]. Briefly, adult male Wistar rats (300–350 g) were anesthetized with intraperitoneal thiopentone sodium (60 mg kg^{-1}), tracheostomized, and mechanically ventilated (75 strokes min^{-1}, tidal volume = 8 ml kg^{-1}, inspired fraction of oxygen=0.21) using a Harvard Model 683 rodent respirator (Harvard

Apparatus, Holliston, MA). Core temperature measured via a rectal probe was maintained at 37°C. Following a left thoracotomy at the fourth intercostal space, the pericardium was opened and the left anterior descending (LAD) coronary artery was ligated just below the left atrial appendage by an intramural 5.0 silk suture and a snare occluder. Immediate palor of the left ventricular free wall was indicative of successful occlusion. After 45 min of myocardial ischemia, reperfusion was allowed for 2 h by removing the coronary occluder.

Rats exposed to ischemia and reperfusion were randomly assigned to treatment with MnTBAP (Sigma Chemicals), 1 mg/kg given 20 min before reperfusion, 30 and 60 min after reperfusion (intraperitoneally), or vehicle only (isotonic saline, 1 ml). The dose of MnTBAP was chosen on the basis of previous experiments showing that MnTBAP at 1 mg/kg was efficient to reduce the consequences of zymosan-induced shock in the rat [30].

Determination of myocardial infarct size—Area at risk (AAR) and infarct size were determined using the triphenyltetrazolium chloride (TTC)-Evans blue technique, as described [11]. The area at risk and the infarcted area were expressed as percentage values according to conventional methods (AAR/left ventricle and infarcted area/AAR).

Myeloperoxidase assay (MPO)—MPO activity was determined in cardiac tissue homogenates, using tetramethylbenzidine and hydrogen peroxide, as described [31]. Activity was measured spectrophotometrically as the change in absorbance at 650 nm at 37°C, using a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA). Results are expressed as units of MPO activity per gram protein (Bradford assay).

Malondialdehyde assay—Malondialdehyde formation was utilized to quantify lipid peroxidation in heart tissue and measured as thiobarbituric acid-reactive material, as described [31]. The levels of MDA are expressed as nanomole MDA per milligram protein.

Protein extraction and immunoblot detection of nitrotyrosine, cleaved caspase-3, and cleaved PARP—Heart tissue was homogeneized (50 mg/ml) in lysis buffer (TrisHCl 10 mM, NP40 0.5%, NaCl 0.15 M, Na_3VO_4 1 mM, NaF 10 mM, PMSF 1 mM, EDTA 1 mM, aprotinin 10 μg/ml, leupeptin 10 μg/ml, and pepstatin 1 μg/ml). Western immunoblotting (30 μg myocardial proteins) was performed as described above, using appropriate dilutions of anti-caspase-3, anti-cleaved caspase-3, anti-PARP-1, anti-cleaved-PARP-1 (Cell Signaling), and mouse monoclonal anti-nitrotyrosine (Cayman Chemical, Ann Arbor, MI) primary antibodies. The immunoblot signal was visualized using enhanced chemiluminescence (ECL, Amersham Biosciences, Otelfingen, Switzerland). Densitometric analysis of ECL autoradiographs was performed using a Personal Densitometer and TotalLab Software.

Presentation of data and statistical analysis—All graphs present data as means± SE. For in vitro experiments, statistical analysis was performed using ANOVA followed by Dunnett's test to compare the effects of various concentrations of $ONOO^-$ with respect to control conditions. In vivo data were analyzed with ANOVA followed by Bonferroni adjustments for all pairwise comparisons, except from area at risk and infarct size which were analyzed with Student's *t* test.

Results

Cytotoxicity of $ONOO^-$ in cultured cardiomyocytes

$ONOO^-$ reduces H9C2 viability—As illustrated in Fig. 1, $ONOO^-$ moderately, but significantly, reduced cell viability 4 h after stimulation (Fig. 1A), at concentrations of 100–

500 μM. At 6 h poststimulation (Fig. 1B), the decrease in cell viability was much more pronounced, an effect that was significant at all concentrations of $ONOO^-$ tested. DP did not have any effect on cell survival.

Apoptotic and necrotic cell death elicited by $ONOO^-$—At 6 h post $ONOO^-$ stimulation, there was a 2-fold increase in LDH release in the medium of cells treated with 250 and 500 μM $ONOO^-$, whereas no significant increase of LDH release was detected in cells exposed to DP and to $ONOO^-$ at 50 and 100 μM (Fig. 1C). As illustrated in Fig. 1D, a marked increase in DNA fragmentation was detected 6 h after treatment with $ONOO^-$ at all concentrations tested. Hoechst 33342 staining clearly indicated that $ONOO^-$ induced nuclear morphological changes typical of apoptosis, an effect that was most pronounced in cells treated with 500 μM $ONOO^-$ (Fig. 2). Several cells with typical apoptotic nuclei also stained positive with PI, which indicates that these cells were at a late stage of apoptosis ("secondary necrosis"), where membranes become permeable to PI [27]. In comparison to apoptotic cell death, necrosis appeared much less pronounced, as only a few cells disclosed morphologically normal nuclei positively stained with PI. Overall, these data indicate that $ONOO^-$ induced both apoptosis and necrosis in H9C2 cardiomyocytes, with apoptosis representing the preferred mode of cell demise in the conditions of our study.

$ONOO^-$ triggers the cleavage of caspase-3 and PARP in H9C2 cells—Further substantiating the proapoptotic role of $ONOO^-$ in H9C2 cells, $ONOO^-$ (but not DP) markedly activated the cleavage of caspase-3 at 4 and 6 h after stimulation at all concentrations tested (Fig. 3). We did not notice any significant change in the expression levels of procaspase-3 in all the conditions. In addition to caspase-3 cleavage, $ONOO^-$ also activated the cleavage of PARP, a well-known substrate of caspase-3, at 4 and 6 h, an effect concomitant to a decrease in the expression levels of full-length PARP (Fig. 3). After 4 h, this effect was noted at 250 and 500 μM (and, to a lesser extent, at 100 μM), while at 6 h poststimulation, all concentrations of $ONOO^-$ clearly induced the cleavage of PARP. In contrast to the effects of authentic $ONOO^-$, DP did not induce the cleavage of PARP and did not affect the expression levels of full-length PARP.

Cytotoxicity of $ONOO^-$ in the myocardium in vivo

Reduction of infarct size by MnTBAP, an $ONOO^-$ scavenger—As shown in Fig. 4A, the area at risk was comparable between the two groups of rats (in % of the left ventricle: control group 47±4%, MnTBAP group 43±4%, P=0.42, t test). Infarct size was markedly and significantly reduced in the animals treated with MnTBAP (in % of the AAR: control group 43±7%, MnTBAP group 13±4%, P=0.0026, t test; in % of the left ventricle: control group, 21 ±4%, MnTBAP group 5±1%, P=0.0067, t test). Fig. 4B presents sections (mid left ventricles) of hearts stained with Evans blue and TTC, which illustrate the reduction of infarct size produced by MnTBAP.

MnTBAP reduces myocardial oxidative stress and neutrophil infiltration—When compared to sham rats (n=3), myocardial ischemia-reperfusion (MIR group, n=5) induced a significant increase in the myocardial content of MDA, a marker of lipid peroxidation, as illustrated in Fig. 5A. This increase was suppressed by treatment with MnTBAP (MIR/MnTBAP group, n = 5), confirming that the latter prevented the development of oxidative stress in the reperfused myocardium. A further consequence of reperfusion was the large rise of myocardial MPO activity, an indicator of neutrophil infiltration (Fig. 5B), an increase significantly blunted by treatment with MnTBAP.

Ischemia-reperfusion induces the formation of nitrotyrosine in the myocardium, which is alleviated by MnTBAP—Myocardial samples obtained from sham rats disclosed

the presence in low amounts of nitrated proteins of approximately 25–30 kDa as shown in Fig. 5C. Following ischemia-reperfusion, a marked increase in the formation of nitrotyrosine (3-NT) was noted, with several major bands located at 45–50 and 25–30 kDa, which were markedly reduced by treatment with MnTBAP. In fact, as indicated by densitometric analysis, while ischemia-reperfusion significantly increased 3-NT formation within the myocardium, there was no statistically significant difference in the amount of nitrated proteins in the heart of sham rats and ischemia-reperfusion rats treated with MnTBAP (Fig. 5D).

MnTBAP suppresses the cleavage of caspase-3 and PARP in the reperfused myocardium—Ischemia followed by reperfusion of the myocardium (MIR group, *n*=8) induced a marked activation of caspase-3, as indicated by a 17-fold increase of the ratio between cleaved caspase-3 and procaspase-3 (Fig. 6A), associated with a 3.5 increase in the ratio of cleaved PARP to full-length PARP (Fig. 6B), when compared to sham rats (*n*=6), indicating that reperfusion elicited a significant activation of apoptotic pathways in the myocardium. Scavenging of $ONOO^-$ with MnTBAP (MIR/MnTBAP group, *n*=9) suppressed the cleavage of both caspase-3 and PARP, as illustrated by densitometric analysis of the immunoblot signals.

Discussion

Myocardial generation of $ONOO^-$ represents an important pathophysiological process in conditions as various as myocardial infarction [2,32], heart failure [33], and cardiomyopathy associated with sepsis [34,35], diabetes [36], and anthracycline therapy [37,38]. In such conditions, $ONOO^-$ plays key roles in the development of myocardial mechanical dysfunction through mechanisms involving the activation of matrix metalloproteinase 2 (MMP-2) [4], the impairment of mitochondrial high-energy phosphate generation [39], the nitration of α-actinin [5], the inactivation of myofibrillar creatine kinase [40], and the inactivation of CaATPase via the nitration of SERCA2a [41]. Furthermore, $ONOO^-$ has been shown to represent an important trigger of myocardial necrosis, by inducing oxidative DNA damage leading to the activation of the DNA repair enzyme PARP, which consumes cellular NAD+, ultimately leading to ATP depletion and necrotic cell death [9,42].

In the present study, we provide the novel evidence that $ONOO^-$ also produces an alternate mode of cardiomyocyte cell death which presents all the characteristics of apoptosis, as indicated by DNA fragmentation (Fig. 1D), the development of apoptotic nuclear morphological alterations (Fig. 2), and the proteolytic activation of caspase-3 and the cleavage of PARP (Fig. 3). In fact, apoptosis, rather than necrosis, appeared as the preferential mode of cardiomyocyte death elicited by $ONOO^-$in the conditions of this study, as $ONOO^-$ evoked a large increase in the markers of apoptosis, but only a modest increase of biological indicators of necrotic cell death (PI staining and LDH release, Figs. 1C and 2). Although a previous study indicated that $ONOO^-$ could induce apoptosis in neonatal and adult rat ventricular myocytes through a mechanism involving an increase in the proapoptotic protein Bax [17], our observation that $ONOO^-$ triggers PARP cleavage has not been previously reported. It must be stressed that PARP activation and PARP cleavage are fundamentally distinct events, linked to completely different modes of cell demise, that is, either necrotic or apoptotic. The cleavage of PARP into 89- and 24-kDa fragments inactivates the enzyme by destroying its ability to respond to DNA strand breaks, thereby preventing the loss of cellular ATP associated with PARP activation and maintaining the cellular energy essential for the execution of apoptosis [43,44]. As such, PARP cleavage functions as a molecular switch between apoptotic and necrotic modes of cell death [14]. Our observations clearly indicate that $ONOO^-$should not be solely regarded as a trigger of PARP activation, but also a potent inducer of the cleavage of this important regulator of cell death in cardiac muscle cells.

In support of our in vitro findings, we identified $ONOO^-$ generation as an important mechanism activating proapoptotic signaling cascades in the heart during myocardial ischemia-reperfusion in vivo. MIR was associated with significant oxidative stress (enhanced levels of malondialdehyde, Fig. 5A), together with the generation of nitrotyrosine (Figs. 5C and 5D), an indirect marker of nitrative stress and more specifically $ONOO^-$ generation [45,46], in the myocardium, in agreement with previously published data [11,47–49]. These changes were accompanied by significant cardiomyocyte apoptosis, as evidenced by the appearance of cleavage fragments of both caspase-3 (Fig. 6A) and PARP (Fig. 6B) in myocardial samples obtained from the reperfused left ventricle. Treatment with MnTBAP significantly reduced MDA formation and nitrotyrosine generation, implying that this compound efficiently alleviated $ONOO^-$ mediated oxidative stress in the myocardium. In parallel, a remarkable effect of MnTBAP was the suppression of the cleavage of both caspase-3 and PARP, associated with a considerable prevention of myocardial injury, as shown by the large reduction of infarct size provided by MnTBAP (Fig. 4). It is here noteworthy that a previous experimental study also reported a marked reduction of infarct size in rats treated with the peroxynitrite decomposition catalyst FP15 [37]. Overall, these data imply that $ONOO^-$ dependent oxidant stress is instrumental in activating proapoptotic signals (caspase-3 and PARP cleavage) in the infarcted heart. Most probably, PARP cleavage consecutive to $ONOO^-$ generation was secondary to the activation of caspase-3, but additional mechanisms may have been implicated as well. Notably, a recent study has demonstrated that PARP can be cleaved in the nucleus of cardiomyocytes through the action of MMP-2 [50], known to be activated by $ONOO^-$ [4]. Whether this pathway also takes place in vivo will require further investigations.

It is important to note that apoptosis has been recently proven to represent a dominant form of cardiomyocyte death in ischemia-reperfusion, and myocardial apoptosis has been suggested as the initiating factor of postinfarction left-ventricular remodeling (see [15,16] for review). Although oxidants and free radicals are considered important triggers of myocardial apoptosis in such conditions, the exact apoptotic stimulus still remains elusive [16]. Furthermore, apoptosis is also critically involved in the cardiac damage associated with anthracycline therapy, diabetes, sepsis, and dilated cardiomyopathy [16], which are all associated with an enhanced generation of $ONOO^-$ within the myocardium [3]. On the basis of our findings, we propose that $ONOO^-$ may represent a major oxidant species involved in the process of cardiomyocyte apoptosis in these cardiac diseases, and that the beneficial effects of anti-$ONOO^-$ therapies reported in these conditions might be related to the prevention of cardiomyocyte apoptosis, an issue which should be critically investigated in future studies.

In addition to its effects on caspase-3 and PARP cleavage, MnTBAP also markedly reduced the myocardial accumulation of polymorphonuclear cells (Fig. 5B), as attested by the significant reduction of cardiac MPO activity, an effect that certainly contributed to a large extent to reduce cardiac injury. Indeed, phagocyte infiltration has been proposed as a key mechanism triggering postreperfusion cardiac damage [49]. These results imply a causative role of $ONOO^-$ on the sequestration of neutrophils within the reperfused myocardium. This hypothesis is supported by previous experimental evidence indicating that $ONOO^-$ favors neutrophil-endothelial interactions by inducing acute endothelial damage and dysfunction [51,52], and by enhancing the expression of various adhesion molecules [53,54]. Although not directly tested in the present work, the marked effect of MnTBAP on MPO activity suggests that similar mechanisms were set in motion by $ONOO^-$ in the myocardium exposed to ischemia and reperfusion.

In summary, our data demonstrate that $ONOO^-$ triggers the activation of caspase-3 and the cleavage of PARP both in cardiomyocytes in vitro and in the reperfused myocardium in vivo. These results contrast with the usual paradigm that $ONOO^-$ is essentially an inducer of cell necrosis through PARP activation, and provide novel information on the mechanisms of

$ONOO^-$ dependent cytotoxicity in the heart. Since $ONOO^-$ is formed in many different cardiac pathologies, in which apoptotic cell death is an important mechanism of myocyte loss, our results support the assertion that $ONOO^-$ may be instrumental in the development of apoptosis in such conditions.

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Abbreviations

MIR
myocardial ischemia-reperfusion

PARP
poly(ADP-ribose) polymerase

MnTBAP
Mn(III)-tetrakis(4-benzoic acid) porphyrin

DMEM
Dulbecco's modified Eagle's medium

FBS
fetal bovine serum

MTT
3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PMSF
phenylmethylsulfonyl fluoride

SDS-PAGE
sodium dodecyl sulfate-polyacrylamide gel electrophoresis

PI
propidium iodide

AAR
area at risk

TTC
triphenyltetrazolium chloride

MPO
myeloperoxidase assay

3-NT
3-nitrotyrosine

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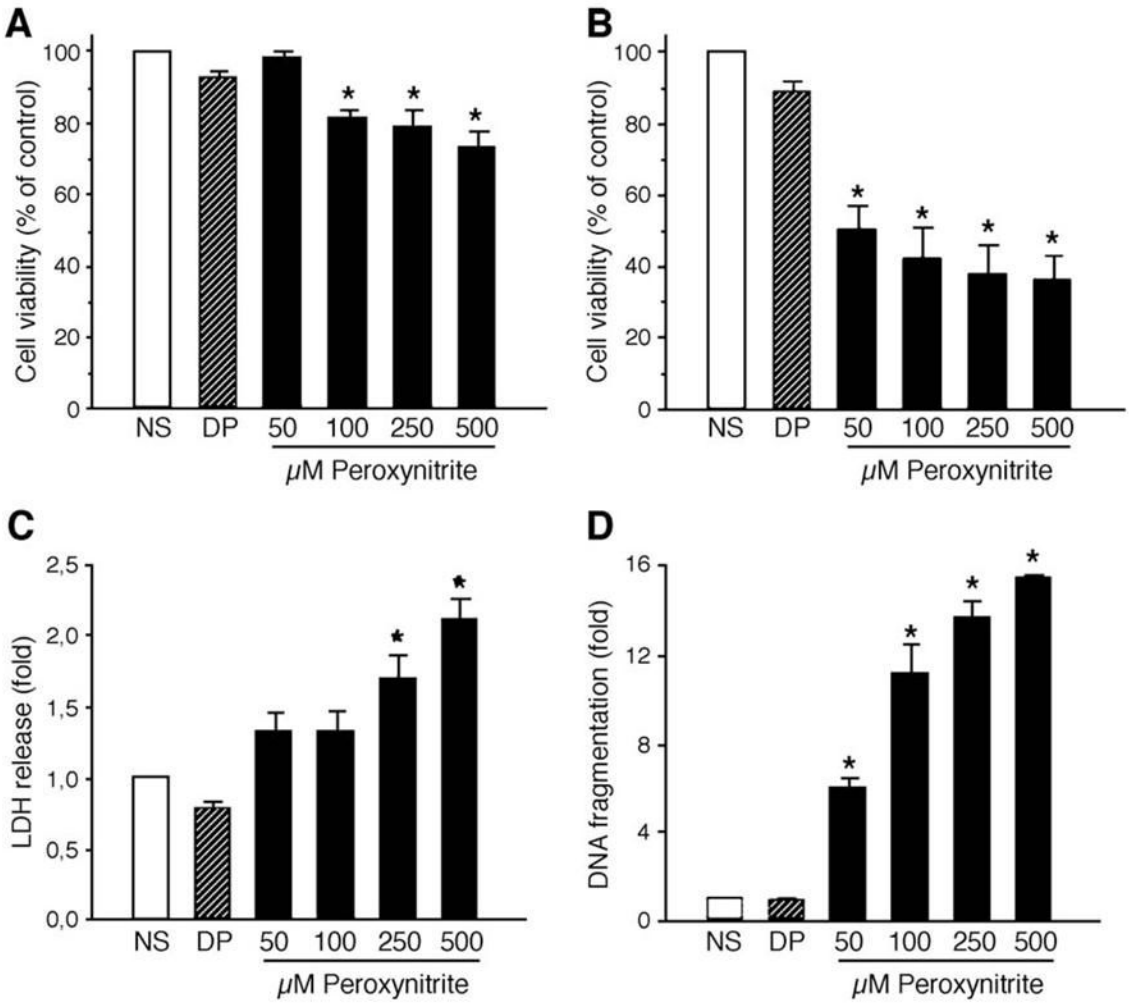

Fig. 1.

Cytotoxicity of $ONOO^-$ in H9C2 cells. (A,B) H9C2 cells were exposed to authentic $ONOO^-$ (50–500 μM) or decomposed $ONOO^-$ (DP) for 20 min and then placed in complete medium for 4 (A) or 6 h (B). Cell viability was determined by the MTT assay, and is expressed in percentage of control, unstimulated cells (NS). While DP had no effect, $ONOO^-$ significantly reduced cell viability at 4 and 6 h. (C,D) H9C2 cells were exposed to $ONOO^-$ or DP for 20 min and then observed for 6 h. LDH release in the medium was measured as an index of cell necrosis. Apoptotic DNA fragmentation was quantified by a specific ELISA (see Materials and methods). $ONOO^-$ elicited a modest, but significant increase in LDH release (expressed in fold augmentation with respect to control cells), only at 250 and 500 μM (C). $ONOO^-$ at all concentrations induced a significant increase in DNA fragmentation, expressed in percentage of control cells (D). Decomposed $ONOO^-$ (DP) did not induce LDH release and DNA fragmentation. Data are means±SE of n=5–6 determinations per condition. *P<0.05 vs. NS (ANOVA followed by the Dunnett test).

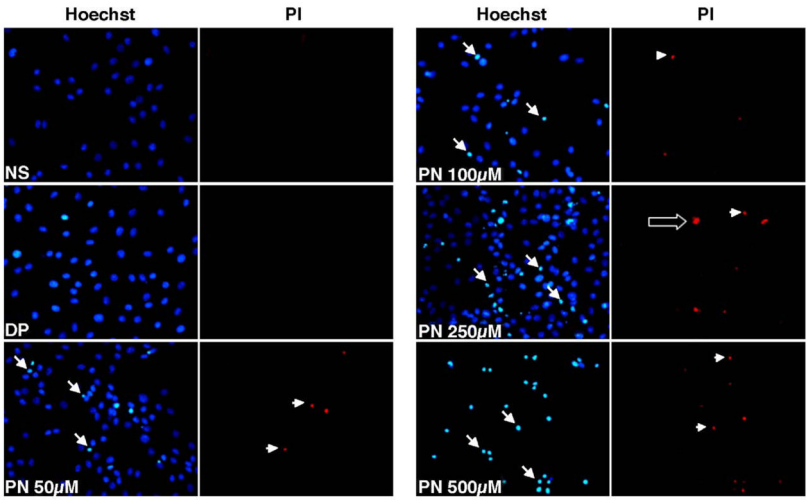

Fig. 2.

Typical apoptotic nuclear morphologic changes are induced by $ONOO^-$ in H9C2 cells. Cells were treated with $ONOO^-$ for 20 min and then placed in complete medium for 6 h. Cells were stained with Hoechst 33342 and PI to examine nuclear morphology. $ONOO^-$ (but not decomposed $ONOO^-$, DP) induced morphological changes characteristic of apoptosis (chromatin condensation and nuclear shrinkage) at all concentrations, most notably at 250 and 500 μM, as indicated by the arrows. Only a small number of cells disclosed intact nuclei and positive PI staining, indicative of primary cell necrosis (large arrow), whereas several cells with apoptotic nuclei were also stained with PI, indicating late apoptosis or secondary necrosis (arrowheads).

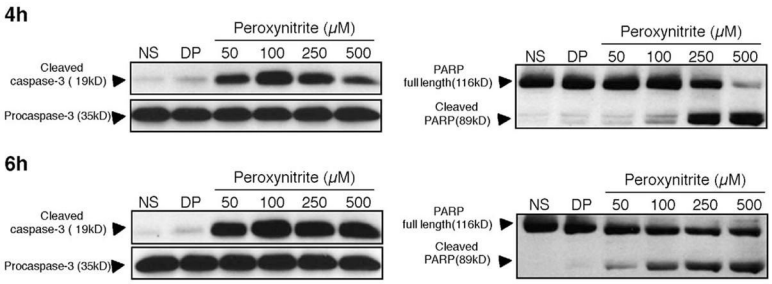

Fig. 3.

ONOO$^-$ activates caspase-3 and triggers the cleavage of PARP in H9C2 cells. Immunoblot detection of procaspase 3 and cleaved caspase-3 (left), full-length and cleaved PARP (right) in cells stimulated with the indicated concentrations of ONOO$^-$, or decomposed ONOO$^-$ (DP) for 20 min, and placed in medium for 4 or 6 h. Caspase-3 was markedly cleaved in response to ONOO$^-$ at all concentrations, but not DP, both at 4 and 6 h. ONOO$^-$, but not DP also triggered PARP cleavage at 100–500 μM after 4 h and at all concentrations after 6 h, as evidenced by an increased signal for cleaved PARP associated to a reduced expression of full-length PARP. The blots are representative of at least 3 independent experiments.

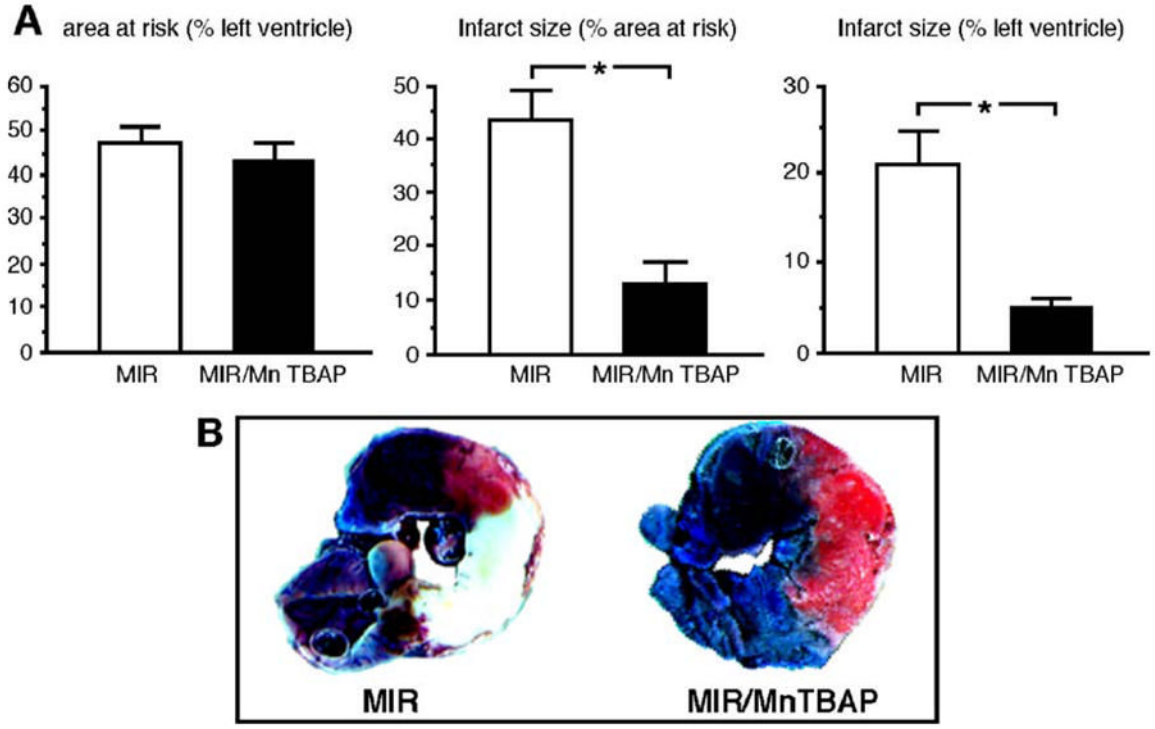

Fig. 4.

The $ONOO^-$ scavenger MnTBAP reduces infarct size in the rat. (A) Area at risk (AAR) and infarct size (expressed in % of AAR or % of left ventricle, LV) in rats exposed to 45 min LAD occlusion followed by 2 h reperfusion. Rats were treated with either MnTBAP (MIR/MnTBAP) or vehicle only (MIR). While AAR was comparable in both groups, there was a significant reduction in infarct size in rats treated with MnTBAP. Means±SE of n=7–8 rats/group. *P<0.05, unpaired t test. (B) Representative sections (mid-left ventricle) of reperfused hearts stained with Evans blue/TTC showing reduced infarct size produced by MnTBAP.

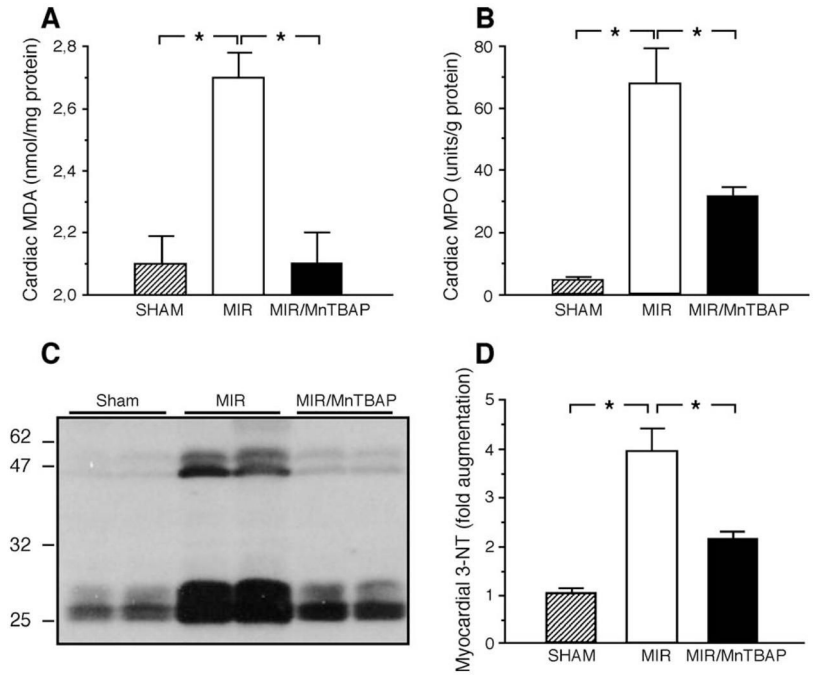

Fig. 5.

MnTBAP reduces MDA formation and MPO accumulation, and suppresses 3-nitrotyrosine formation in myocardial ischemia-reperfusion. Cardiac MDA formation (A), an index of lipid peroxidation, and MPO activity (B) in sham rats (*n*=3) or in rats exposed to myocardial ischemia-reperfusion, treated with MnTBAP (MIR/MnTBAP group, *n*=5) or saline only (MIR group, *n*=5). MIR induced significant increases of both MDA and MPO, which were significantly reduced by MnTBAP. MDA and MPO were not significantly different between sham rats and MIR/MnTBAP rats. (C) Representative Western blots of 3-nitrotyrosine (3-NT) within myocardial samples obtained from the three groups of rats (*n*=4 rats/group). Molecular weight markers are indicated on the left. (D) Densitometric analysis shows that MnTBAP significantly prevented 3-NT formation upon myocardial reperfusion. There was no significant difference between sham and MIR/MnTBAP rats. Means±SE. **P*<0.05 (ANOVA followed by Bonferroni).

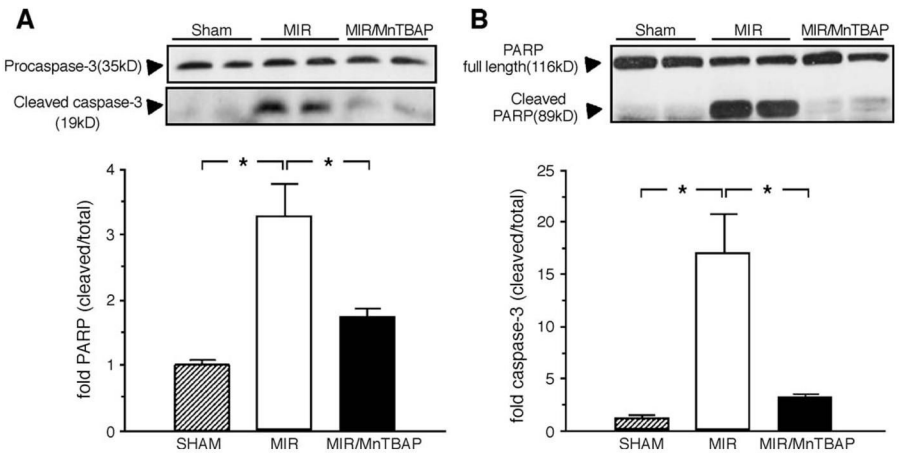

Fig. 6.

MnTBAP prevents the myocardial cleavage of caspase-3 and PARP during myocardial infarction. Representative Western blots of procaspase 3 and cleaved caspase-3 (A), and of full-length and cleaved PARP (B), in myocardial tissue of sham rats and rats exposed to myocardial ischemia-reperfusion, treated with MnTBAP (MIR/MnTBAP) or saline (MIR). The bar graphs show densitometric analysis of the blots, revealing a significant reduction of the cleavage of caspase-3 and PARP in hearts from rats treated with MnTBAP. There was no significant difference between sham and MIR/MnTBAP rats Means±SE of n=6–9 rats/group. *P<0.05 (ANOVA followed by Bonferroni).