Poly(ADP-Ribose) Polymerase Inhibition Improves Endothelial Dysfunction Induced by Hypochlorite

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Reactive oxygen species, such as myeloperoxidase-derived hypochlorite, induce oxidative stress and DNA injury. The subsequent activation of the DNA-damage–poly(ADP-ribose) polymerase (PARP) pathway has been implicated in the pathogenesis of various diseases, including ischemia-reperfusion injury, circulatory shock, diabetic complications, and atherosclerosis. We investigated the effect of PARP inhibition on the impaired endothelium-dependent vasorelaxation induced by hypochlorite. In organ bath experiments for isometric tension, we investigated the endothelium-dependent and endothelium-independent vasorelaxation of isolated rat aortic rings using cumulative concentrations of acetylcholine and sodium nitroprusside. Endothelial dysfunction was induced by exposing rings to hypochlorite (100–400 μM). In the treatment group, rings were preincubated with the PARP inhibitor INO-1001. DNA strand breaks were assessed by the TUNEL method. Immunohistochemistry was performed for 4-hydroxynonenal (a marker of lipid peroxidation), nitrotyrosine (a marker of nitrosative stress), and poly(ADP-ribose) (an enzymatic product of PARP). Exposure to hypochlorite resulted in a dose-dependent impairment of endothelium-dependent vasorelaxation of aortic rings, which was significantly improved by PARP inhibition, whereas the endothelium-independent vasorelaxation remained unaffected. In the hypochlorite groups we found increased DNA breakage, lipidperoxidation, and enhanced nitrotyrosine formation. The hypochloride-induced activation of PARP was prevented by INO-1001. Our results demonstrate that PARP activation contributes to the pathogenesis of hypochlorite-induced endothelial dysfunction, which can be prevented by PARP inhibitors. Exp Biol Med 232:1204–1212, 2007

Key words: hypochlorite; oxidative stress; DNA injury; poly(ADP-ribose) polymerase; endothelial dysfunction

Introduction

Reactive oxygen species (ROS) are believed to play a key role in the pathogenesis of vascular dysfunction observed in such processes as ischemia-reperfusion, hypertension, diabetes, atherosclerosis, aging, or inflammation. Vascular and phagocytic NADPH oxidases as well as the electron transport chain of mitochondrial terminal oxidation are the major sources of the oxidants superoxide ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2). Myeloperoxidase (MPO), a heme protein secreted mainly by neutrophil granulocytes, catalyzes the oxidation of chloride ions by H_2O_2, resulting in the production of hypochlorous acid/hypochlorite ($HOCl/OCl^-$), a potent oxidizing and chlorinating species. Through the action of MPO, up to 70% of the generated H_2O_2 is converted to OCl^-, leading to increased toxicity (1).

OCl^- is known to be extremely toxic to mammalian cells. It interacts with plasma membrane components (oxidizing sulfhydryl groups; inactivating membrane transporters, Ref. 2; modifying phospholipids, Ref. 3; and inducing lipid peroxidation, Ref. 4) and contributes to the degradation of matrix proteins (5). Furthermore, it can enter cells and attack intracellular biomolecules (oxidation of sulfhydryl, methionin and tryptophan residues; formation of protein carbonyls, chloramines and aldehydes; chlorination of various proteins and DNA bases), resulting in loss of intracellular ATP and NAD, tissue degradation, and DNA fragmentation (2–3, 6–8).

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DNA strand breaks induced by ROS lead to the activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP), which initiates an energy-consuming metabolic cycle by transferring ADP-ribose units from NAD^+ to nuclear proteins. This process results in rapid depletion of NAD^+ and intracellular ATP pools and impaired mitochondrial respiration, eventually leading to cellular dysfunction, apoptosis, or necrosis (9–11). The ROS–DNA injury–PARP pathway has recently been established as a major downstream intracellular pathway of nitrosative and oxidative stress. According to recent data (12–16), pharmacologic inhibition of PARP has emerged as a novel antioxidant therapeutic possibility in multiple experimental models of disease (11, 17).

Former studies on DNA injury induced by OCl^- showed that low concentrations of OCl^- (<50 μ*M*) evoke mostly base modifications on DNA without inducing strand breaks and cause oxidative inactivation of the PARP enzyme in cultured cells (2, 18); however, recent works reported about superoxide-dependent (19), hydroxyl radical ($^{\cdot}OH$)-dependent (20, 21), and chloramine-dependent (22) damaging mechanisms of higher concentrations (100–500 μ*M*) of OCl^- that can eventually lead to DNA strand breakage. Furthermore, excessive chlorination of DNA by OCl^- promotes the dissociation of the double strand that leads to breakage and degradation of DNA (7, 23).

Several recent studies report endothelial dysfunction (impairment of endothelium-dependent vasorelaxation) induced by $HOCl/OCl^-$ in rabbit arteries, rat aorta, and guinea pig coronary arteries (19, 24–27). The underlying intracellular processes are objects of intensive investigations in recent years. Emerging evidence supports the role of defects in endothelial nitric oxide (NO) production (endothelial nitric oxide synthase [eNOS] inhibition; Refs. 19, 26, 28, 29) and OCl^--induced apoptosis of endothelial cells (6, 30), but the exact molecular mechanisms remain unclear.

Based on these findings we investigated in the present study the involvement of the PARP pathway in the development of vascular dysfunction caused by the reactive oxidant OCl^-. We examined whether the deleterious effects of OCl^- could be prevented or reduced by using INO-1001, the potent pharmacologic inhibitor of the PARP enzyme.

Materials and Methods

Animals. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85–23, revised 1996). All procedures and handling of animals during the investigations were reviewed and approved by the Ethics Committee of the Land Baden-Württemberg for Animal Experimentation.

Three-month-old male Sprague-Dawley rats (250–350 g; Charles River, Sulzfeld, Germany) were housed in a room at a constant temperature of 22°C ± 2°C with 12:12-hr light-dark cycles and were fed a standard laboratory rat diet and water *ad libitum*.

***In Vitro* Organ Bath Experiments.** Rats were anesthetized with sodium pentobarbitone (60 mg/kg). The descending thoracic aorta was carefully removed and quickly transferred to cold (4°C) Krebs-Henseleit solution (118 m*M* NaCl, 4.7 m*M* KCl, 1.2 m*M* KH_2PO_4, 1.2 m*M* $MgSO_4$, 1.77 m*M* $CaCl_2$, 25 m*M* $NaHCO_3$, 11.4 m*M* glucose; pH = 7.4). The aortae were prepared and cleaned from periadventitial fat and surrounding connective tissue and were cut transversely into 4-mm–wide rings ($n = 3$ or 4 from each animal) using an operation microscope.

Isolated aortic rings were mounted on stainless steel hooks in individual organ baths (Radnoti Glass Technology, Monrovia, CA) containing 25 ml Krebs-Henseleit solution at 37°C and aerated with 95% O_2 and 5% CO_2. Special attention was paid during the preparation to avoid damaging the endothelium.

Isometric contractions were recorded using isometric force transducers (Radnoti Glass Technology), digitized, stored, and displayed with the IOX Software System (EMKA Technologies, Paris, France).

The aortic rings were placed under a resting tension of 2 g and equilibrated for 60 mins. During this period, tension was periodically adjusted to the desired level, and the Krebs-Henseleit solution was changed every 30 mins. Potassium chloride (KCl) was used in these experiments to test viability and prepare vessel rings for stable contractions and reproducible dose-response curves to other vasoactive agents. At the beginning of each experiment, maximal contraction forces to KCl (80 m*M*) were determined, and aortic rings were washed until resting tension was again obtained. After that, in the OCl^- groups, endothelial injury was induced by exposing the aortic rings to OCl^- (100, 200, and 400 μ*M*, a dose range used in previous studies; Ref. 19) for 30 mins. In the PARP inhibitor treatment group, 10 mins before addition of OCl^-, rings were preincubated with INO-1001 (1 μ*M*), which was present also during OCl^- exposure. At 30 mins after addition of OCl^-, aortic preparations were rinsed and preconstricted with phenylephrine (PE; 10^{-6} *M*) until a stable plateau was reached, and relaxation responses were examined by adding cumulative concentrations of endothelium-dependent dilator acetylcholine (ACh; 10^{-9} to 10^{-4} *M*) and endothelium-independent dilator sodium nitroprusside (SNP; 10^{-10} to 10^{-5}*M*).

In additional experiments, the potential direct effects of INO-1001 incubation on vascular function were investigated by a similar protocol without OCl^- exposure.

In an additional set of experiments, the measurements of endothelium-dependent vasorelaxation in the experimental groups were performed on aortic rings preincubated with N^G-nitro-L-arginine methyl ester (L-NAME) to confirm that the relaxation in all groups was mediated by NO.

Contractile responses are expressed as grams of tension, and relaxation is expressed as percent of contraction induced by PE (10^{-6} *M*).

Immunohistochemical Analysis. Additional experiments with aortic segments were performed for histologic and immunohistochemical processing. After excision and preparation of the descending thoracic aorta of the rats (as described above), aortic rings were placed in Krebs-Henseleit solution at 37°C, aerated with 95% O_2 and 5% CO_2, and divided into six groups ($n = 5$ in each group) corresponding to the groups in the organ bath experiments as follows: control group (no treatment), OCl^- groups (exposure to 100, 200, or 400 μ*M* OCl^- for 30 mins), OCl^- + INO-1001 group (10-min preincubation with 1 μ*M* INO-1001 and subsequent exposure to 200 μ*M* OCl^- for 30 mins), INO-1001 control group (40 mins incubation with 1 μ*M* INO-1001). Rings were fixed in buffered paraformaldehyde solution (4%) and embedded in paraffin. Three adjacent sections were processed for each of the following types of immunohistochemical labeling.

According to the methods previously described (31), we performed immunohistochemical staining for nitrotyrosine (NT, a marker of nitrosative stress in general), and for poly(ADP-ribose) (PAR, the enzymatic product of PARP). Primary antibodies used for the stainings were polyclonal sheep anti-NT antibody (Upstate, Chicago, IL) and mouse monoclonal anti-PAR antibody (Calbiochem, San Diego, CA).

We performed immunohistochemical staining for 4-hydroxynonenal (4-hydroxy-2-nonenal, HNE; a marker for lipid peroxidation) as follows: Sections were deparaffinized and rehydrated, and then endogenous peroxidase activity was suppressed by treating slides with 0.5% H_2O_2 in methanol for 15 mins. Antigenic epitopes were retrieved by microwaving in 10 m*M* citrate buffer (pH = 6.0). Sections were washed three times in 0.01 *M* phosphate-buffered saline (PBS; pH = 7.4) and blocked with 1.5% normal goat serum (Vector Laboratories, Burlingame, CA) in PBS containing 0.2% Triton X-100 (blocking serum) for 60 mins. Primary antibodies against HNE (Calbiochem) were applied at 1:200 dilution in blocking serum, and sections were incubated overnight at 4°C. After washing in PBS containing 0.2% Triton X-100 (wash buffer), sections were incubated with biotinylated anti-rabbit antibodies (1:200 in blocking serum; Vector Laboratories) for 30 mins. Sections were washed in wash buffer and incubated with ABC peroxidase reagent (Vectastain Elite ABC kit; Vector Laboratories) for 30 mins. After washing in wash buffer, sections were rinsed in PBS, and peroxidase sites were revealed with 3,3′-diaminobenzidine-tetrahydrochloride (DAB) and H_2O_2 (DAB substrate kit; Vector Laboratories). Slides were washed and counterstained with Gill's hematoxylin (Accustain; Sigma Diagnostics, St. Louis, MO), dehydrated in an ascending alcohol series, cleared in xylene, and coverslipped with Permount (Fisher Chemicals, Fairlawn, NJ).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Reaction. TUNEL assay was performed for detection of DNA strand breaks. The detection was carried out using a commercial kit following the protocol provided by the manufacturer (Chemicon International, Temecula, CA). Briefly, aortic segments of all groups were fixed in neutral-buffered formalin and embedded in paraffin. Sections (4 μm thick) were placed on adhesive slides. Rehydrated sections were treated with 20 μg/ml DNase-free Proteinase K (Sigma-Aldrich, Munich, Germany) to retrieve antigenic epitopes, followed by 3% H_2O_2 to quench endogenous peroxidase activity. Free 3′-OH termini were labeled with digoxigenin-dUTP for 1 hr at 37°C using a terminal deoxynucleotidyl transferase (TdT) reaction mixture (Chemicon International). Incorporated digoxigenin-conjugated nucleotides were detected using a horseradish peroxidase–conjugated antidigoxigenin antibody and DAB. Sections were counterstained with Gill's hematoxylin. Dehydrated sections were cleared in xylene, mounted with Permount (Fisher Scientific, Schwerte, Germany), and coverslips were applied.

Based on the intensity and distribution of labeling, semiquantitative histomorphologic assessment was performed using conventional microscopy by experimenters blinded to the treatment groups.

In cases of HNE, NT, and PAR staining, the results were expressed with a scoring system. Based on the staining intensity, specimens were coupled with intensity score values as follows: 0 = no positive staining, 1 to 3 = increasing degrees of intermediate staining, and 4 = extensive staining. According to the amount of positively stained cells, an area score was assigned (1 = up to 10% positive cells, 2 = 11% to 50% positive cells, 3 = 51% to 80% positive cells, 4 = >80% positive cells). Finally, an average score (0–12) for the whole picture was calculated (intensity score multiplied by area score).

For assessment of TUNEL-labeled cells, the number of positive cell nuclei/microscopic examination field (×250 magnification) was counted (four fields characterizing each specimen), and an average value was calculated for each experimental group.

Statistical Analysis. All data are expressed as means ± SEM. Intergroup comparisons were performed using one-way analysis of variance followed by Student's unpaired *t* test with Bonferroni's correction for multiple comparisons. Differences were considered significant when $P < 0.05$.

Drugs. Sodium OCl^- solution (Sigma-Aldrich) was diluted with distilled water. PE, ACh, SNP, and L-NAME (Sigma-Aldrich) were dissolved in normal saline. INO-1001 was dissolved in 5% glucose solution.

Results

TUNEL and Immunohistochemical Analysis. Using the TUNEL assay we found significant DNA breakage in the aortic wall in the OCl^- groups, as reflected

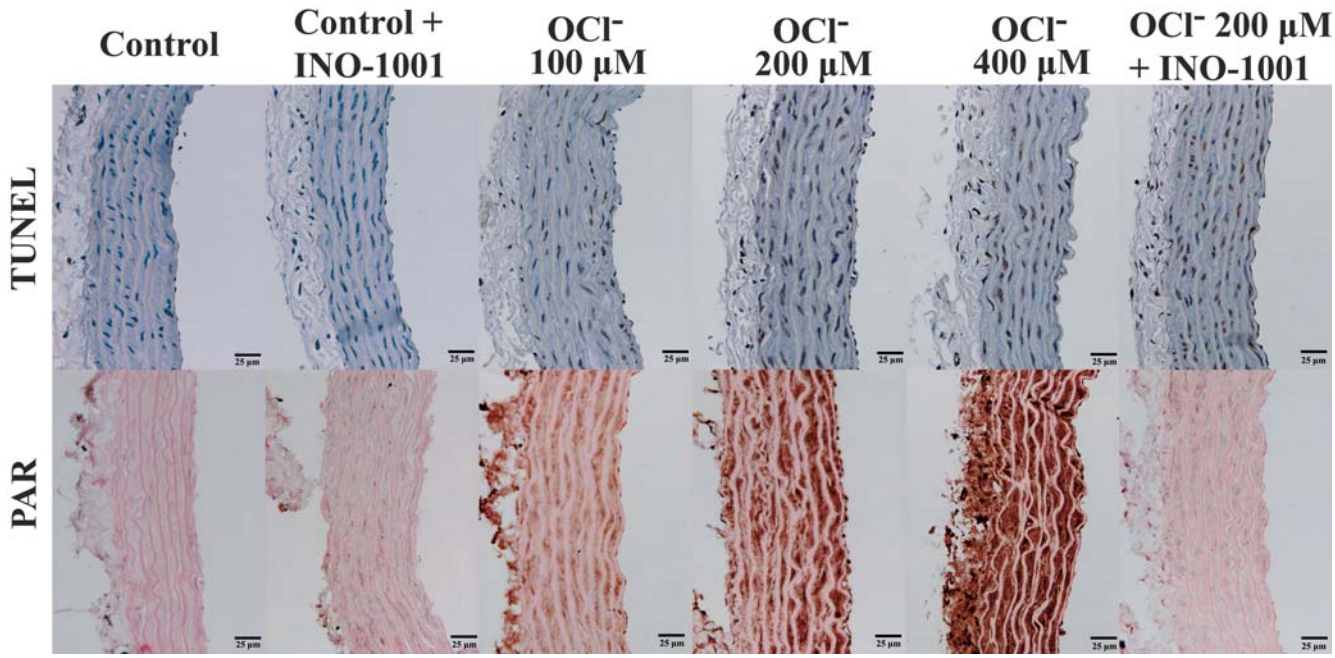

Figure 1. Photomicrographs of TUNEL assay and PAR immunohistochemistry. Representative photomicrographs of TUNEL reaction (upper panel; brown staining in the cell nuclei) and representative immunohistochemical staining for PAR (lower panel; brown staining) in the vessel wall of control, INO-1001–pretreated control, OCl^--exposed (OCl^-: 100, 200, and 400 µ*M*), and INO-1001–pretreated, OCl^--exposed thoracic aortic rings (magnification: ×400; scale bar, 25 µm).

also by the quantitative assessment of TUNEL-positive cells (Figs. 1 and 2A). Pretreatment with INO-1001 did not significantly affect DNA strand breaks induced by OCl^- (Fig. 2A). In turn, as expected, blood vessels not exposed to OCl^- (i.e., the control and the INO-1001 control group) showed essentially no TUNEL positivity. Figure 1 (upper panel) shows representative sections for TUNEL in the different groups.

Immunostaining for both HNE and NT showed enhanced immunoreactivity in the OCl^- groups that reaches the level of significance at the OCl^- concentration of 400 µ*M*. Pretreatment of the rings with INO-1001 did not significantly affect the immunoreactivity for these markers (Fig. 2C and D and Fig. 3). Figure 3 shows representative sections for HNE (upper panel) and for NT (lower panel) in the different groups.

As shown in Figure 2B, a marked degree of PARP activation was observed in the aortic wall sections of the OCl^- groups compared with controls, as evidenced by higher PAR scores. In the case of 100 µ*M* OCl^-, we found a tendency toward increased PAR immunoreactivity, which reached statistical significance in the 200 µ*M* and 400 µ*M* OCl^- groups. Pretreatment with INO-1001 resulted in significantly reduced PAR formation in the aortic rings exposed to 200 µ*M* OCl^- (Figs. 1 and 2B), whereas it had no effect on control rings. Figure 1 (lower panel) shows representative staining for PAR in the different groups.

Vascular Function. Regarding the contractions of aortic rings exposed to PE (10^{-6} *M*), the groups exposed to OCl^- showed enhanced maximal contraction forces (100 µ*M* OCl^- vs. 200 µ*M* OCl^- vs. 400 µ*M* OCl^-: 5.89 ± 0.10 g vs. 6.29 ± 0.09 g vs. 6.33 ± 0.11 g, respectively; $P < 0.05$) compared with controls (5.06 ± 0.10 g).

A dose-dependent impairment of endothelium-dependent vasorelaxation caused by OCl^- was demonstrated in our *in vitro* organ bath experiments. The endothelial dysfunction induced by the reactive oxidant OCl^- was indicated by the reduced maximal relaxation of isolated aortic rings to ACh (control vs. 100 µ*M* OCl^- vs. 200 µ*M* OCl^- vs. 400 µ*M* OCl^-: 86.21% ± 1.574% vs. 75.39% ± 2.891% vs. 66.57% ± 3.137% vs. 54.99% ± 4.054%, respectively; $P < 0.05$) and the OCl^- dose-dependent rightward shift of the dose-response curve compared with the control group (Fig. 4A). The endothelium-independent vascular smooth muscle function indicated by the vasorelaxation of aortic rings to SNP was not impaired by OCl^- (Fig. 4B).

Inhibition of the PARP enzyme by INO-1001 significantly enhanced the ACh-induced, endothelium-dependent, NO-mediated vasorelaxation after exposure with 200 µ*M* OCl^- (maximal relaxation: 200 µ*M* OCl^- + INO-1001 vs. 200 µ*M* OCl^-: 78.74% ± 3.142% vs. 66.57% ± 3.137%, respectively; $P < 0.05$), indicating improved endothelial function (Fig. 5A). The same pretreatment had no significant effect on PE contraction (200 µ*M* OCl^- + INO-1001 vs. 200 µ*M* OCl^-: 6.10 ± 0.09 g vs. 6.29 ± 0.09 g) or on the endothelium-independent vasorelaxation of aortic rings to SNP (Fig. 5B).

In the INO-1001 control group we found no alterations in the contraction and in the ACh- and SNP-induced vasorelaxation compared with control (data not shown).

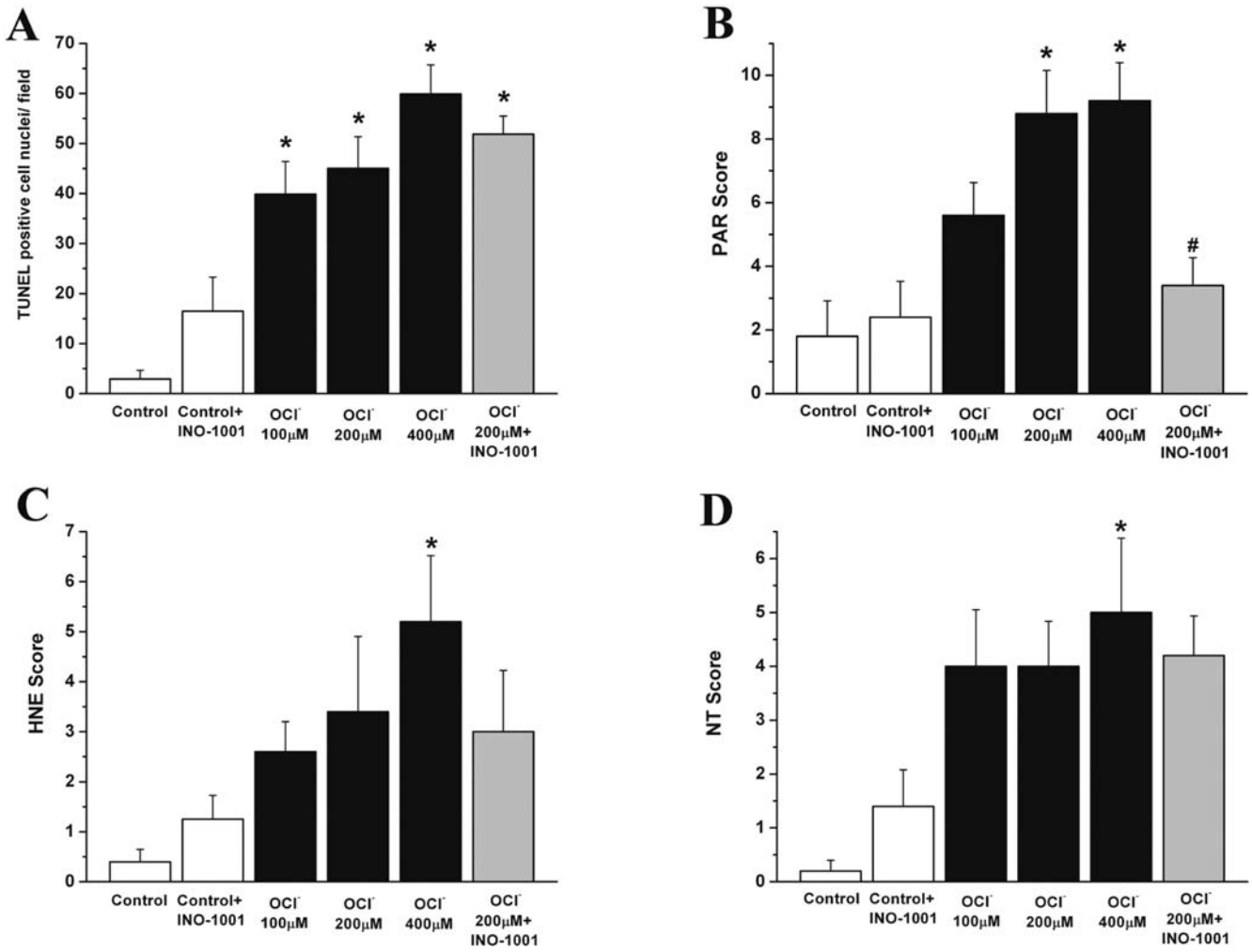

Figure 2. Scoring of TUNEL assay and PAR, HNE, and NT immunohistochemistry. Average number of TUNEL-positive cell nuclei in a microscopic field (magnification: ×250) of the aortic wall of control, INO-1001–pretreated control, OCl^--exposed (OCl^-: 100, 200, and 400 μ*M*), and INO-1001–pretreated, OCl^--exposed thoracic aortic rings (A). Immunohistochemical scores for PAR (B), HNE (C), and NT (D) in the aortic wall in the different groups. **P* < 0.05 versus control; #*P* < 0.05 versus OCl^- 200 μ*M*.

The ACh-induced, endothelium-dependent vasorelaxation was fully abolished in all groups of aortic rings preincubated with L-NAME (data not shown).

Discussion

$OCl^-$and OCl^--Induced Vascular Changes. Reactive oxygen species, such as HOCl/OCl^-, play a double-faced role in the physiology and pathophysiology of the human organism. They are considered to be crucial in the host defense mechanisms of neutrophil granulocytes against bacteria and fungi; on the other hand, overproduction of free radicals and reactive oxidants are implicated in the development of several diseases. In the field of cardiovascular disorders, ROS play a pathophysiologic role, among others, in atherosclerosis, hypertension, ischemia-reperfusion, cardiovascular aging, restenosis, and diabetic cardiovascular complications (32).

Under pathophysiologic conditions (e.g., inflammation or ischemia-reperfusion), high levels of OCl^- (up to the millimolar concentration range) can be reached in the local circulation of the affected area (25). Therefore, the effects of OCl^- on the vasculature has been the subject of intensive investigations in recent years (19, 24–26, 33, 34). Several studies report that *in vitro* exposure of vessels to HOCl results in impaired function of the endothelium (19, 24, 25). In accordance with these results, we report in the present study impaired endothelium-dependent, ACh-induced relaxation of isolated rat aortic rings exposed to 100, 200, and 400 μ*M* HOCl. The endothelium-independent relaxation induced by the exogenously administered NO donor SNP was unaffected by these concentrations of OCl^-, indicating normal dilative capacity of the vascular smooth muscle. These functional data are consistent with the results of Stocker *et al*. on rabbit aortic rings incubated *in vitro* in HOCl (50–500 μ*M*; Ref. 19). In contrast, another study reported inhibition of endothelium-dependent vasorelaxation in rat aorta already at lower concentrations of HOCl (1–50 μ*M*); however, at a longer incubation time (25).

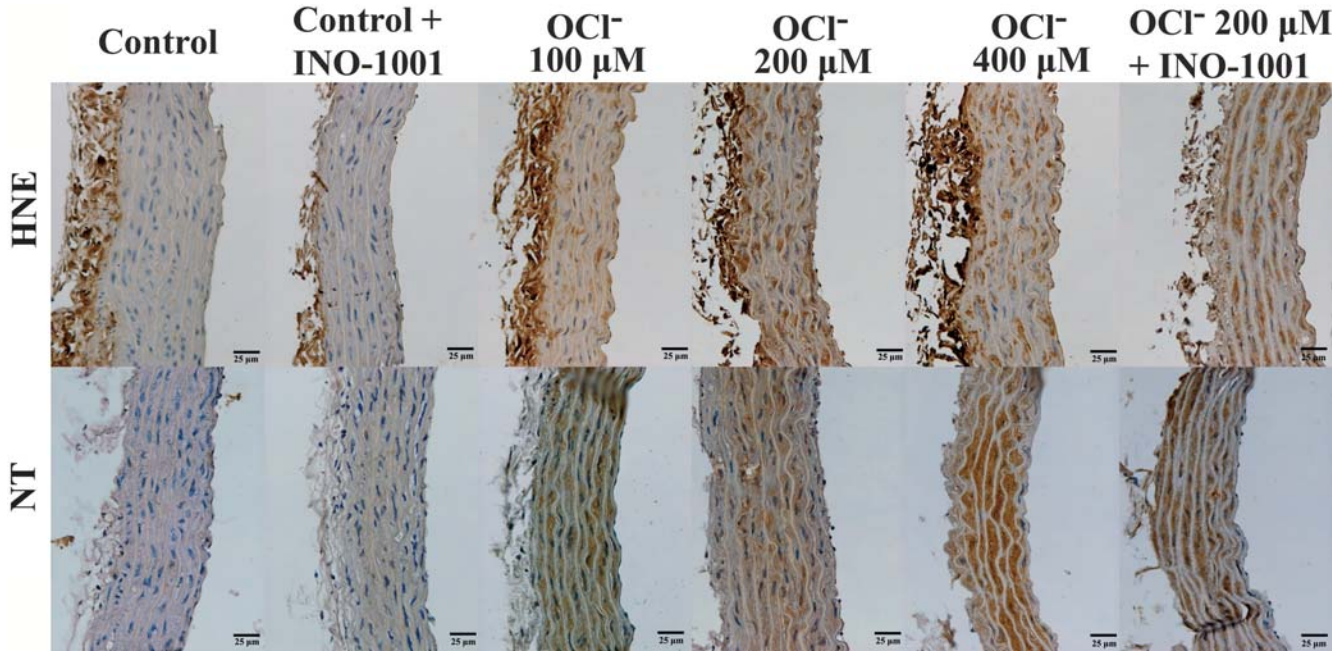

Figure 3. Photomicrographs of HNE and NT immunohistochemistry. Representative immunohistochemical stainings for HNE (upper panel; brown staining) and for NT (lower panel; brown staining) in the vessel wall of control, INO-1001–pretreated control, OCl^--exposed (OCl^-: 100, 200, and 400 µ*M*), and INO-1001–pretreated, OCl^--exposed thoracic aortic rings (magnification: ×400, scale bar, 25 µm).

Similarly, Leipert *et al*. found impaired coronary endothelial function in isolated guinea pig hearts preperfused with HOCl (27). We found an increase in contraction forces induced by PE in the OCl^--exposed groups, which might be due to OCl^--induced impairment of basal NO production of the endothelium or alterations in receptor density and/or receptor/effector coupling.

While the phenomenon of endothelial dysfunction induced by OCl^- is well described, the underlying intracellular pathways and exact molecular mechanisms are still not fully understood.

There is evidence that OCl^- decreases the bioactivity of endothelial NO in cultured endothelial cells (19, 28). HOCl-mediated chlorination of L-arginine (substrate for eNOS; Ref. 26) and HOCl-modified, low-density lipoprotein (LDL; Ref. 35) can impair endothelial NO synthesis; furthermore, HOCl decreases NO production in endothelial cells by uncoupling eNOS (19). Recent works suggest an important role for other oxidants and free radicals in the molecular mechanisms of OCl^--mediated vascular injury. Vascular production of superoxide anions ($O_2^{-\cdot}$) may be stimulated by HOCl *via* eNOS uncoupling and vascular NADPH oxidase activation (19). Superoxide anions interact with the physiologic mediator NO, forming the potent oxidant peroxynitrite ($ONOO^{-\cdot}$). Free hydroxyl radicals ($^{\cdot}OH$) and singlet oxygen can be formed in the reactions of HOCl with iron(II) complexes (36), superoxide anions (20), and H_2O_2 (37). Although these are only minor products of OCl^-, they might contribute to the HOCl-induced vascular damage.

We demonstrated in the present study that exposure of rat aortic rings to OCl^- resulted in formation of DNA strand breaks in the vessel wall, as evidenced by TUNEL assay.

Contrasting these results, previous studies reported no DNA breakage induced by HOCl in cultured cells—only by coincubation with HOCl and H_2O_2 (2, 38). In fact, HOCl has a direct chlorinating and oxidizing effect on DNA, which leads mainly to base modifications, dissociation of the double strand, and degradation of DNA (7). However, indirectly, *via* production of other oxidants and free radicals (as discussed above) and yielding multiple semi-stable chloramines (22), HOCl might induce single- and double-strand breaks on DNA.

Previous works on human leukocytes reported inhibition of PARP by low (10–25 µ*M*) concentrations of HOCl (18). In contrast, our immunohistochemical staining for PAR clearly demonstrates the activation of the PARP enzyme, thus the pathway of ROS–DNA injury–PARP in the vessel wall treated with OCl^-.

For a long time, NT has been considered to be the "footprint of peroxynitrite generation"; however, recent studies revealed that other pathways can also induce tyrosine nitration. HOCl reacts with nitrite (the major end product of NO metabolism) to form nitril chloride ($Cl\text{-}NO_2$), which reacts directly with tyrosine (39). Our present data showing enhanced NT formation in the OCl^- groups are in line with these results: both peroxynitrite and nitril chloride formation may contribute to the immunoreactivity shown for NT.

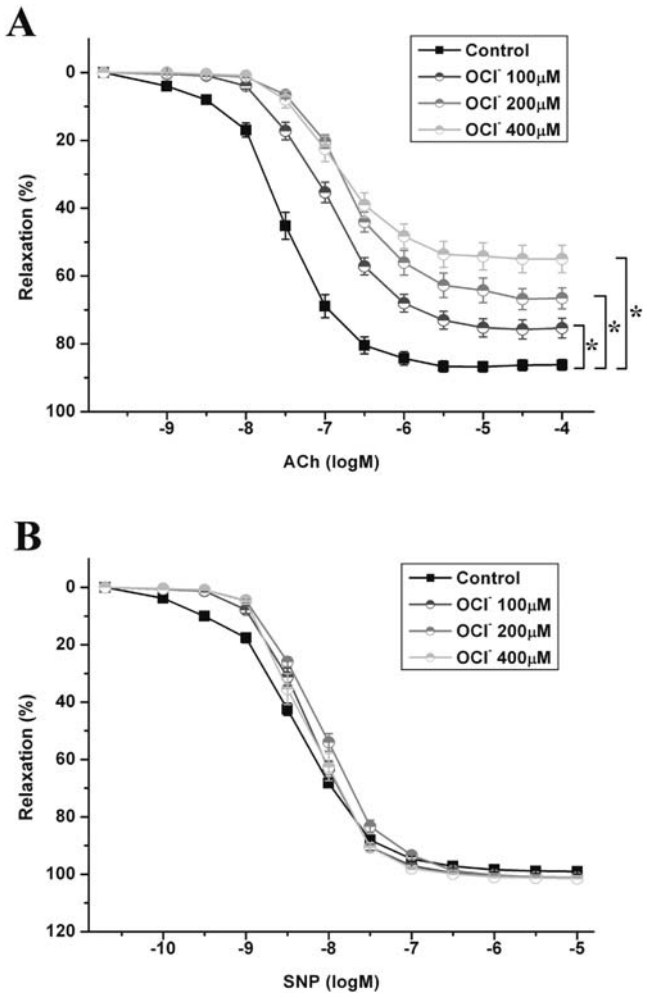

Figure 4. Effects of OCl^- on the vascular function of rat thoracic aortic rings. ACh-induced, endothelium-dependent vasorelaxation (A) and SNP-induced, endothelium-independent vasorelaxation (B) in the control and OCl^--exposed (100, 200, and 400 μM) groups. Each point of the curves represents mean ± SEM of 18–20 experiments in thoracic aortic rings of the different groups. *$P <$ 0.05 versus control.

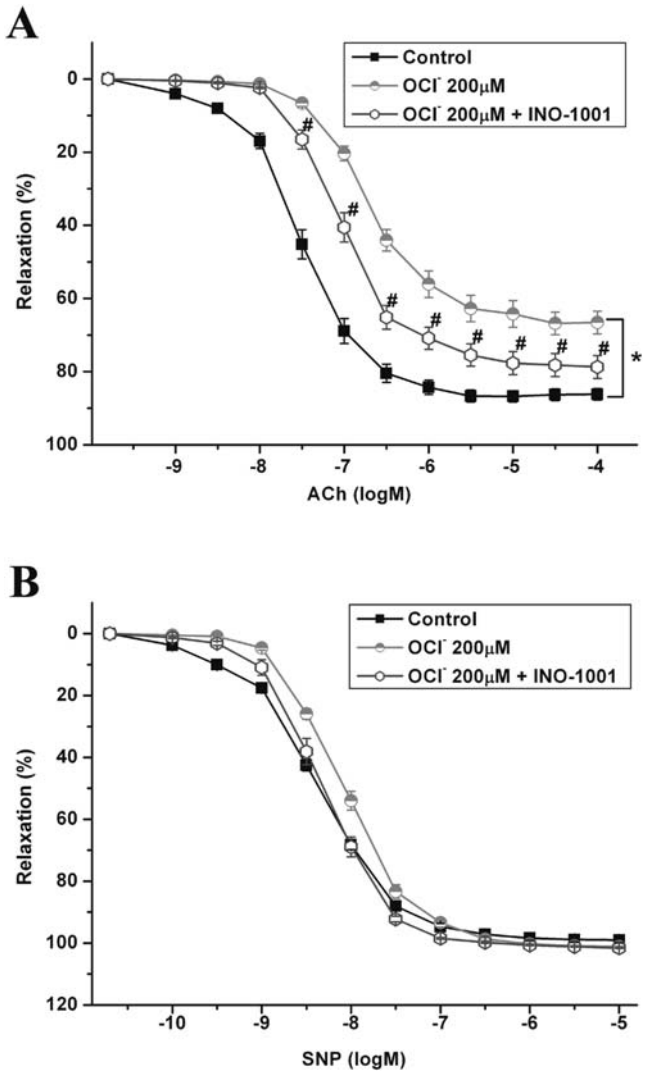

Figure 5. Improvement of OCl^--induced endothelial dysfunction by inhibition of PARP with INO-1001. ACh-induced, endothelium-dependent vasorelaxation (A) and SNP-induced, endothelium-independent vasorelaxation (B) in the groups of control rings, rings exposed to 200 μM OCl^-, and INO-1001–pretreated rings exposed to OCl^- (200 μM). Each point of the curves represents mean ± SEM of 18–20 experiments in thoracic aortic rings of the different groups. *$P <$ 0.05 versus control, #$P <$ 0.05 versus OCl^- 200 μM.

Immunohistochemical staining for HNE showed signs of lipid peroxidation in the aortic wall after OCl^- exposure, which is similar to previous reports on the lipid-modifying actions of HOCl (3, 4).

Effects of PARP Inhibition on OCl^--Induced Vascular Changes: Proposed Underlying Molecular Pathomechanisms. The major finding of the present study is that pharmacologic inhibition of the PARP enzyme (pretreatment of aortic rings with INO-1001) significantly enhanced the endothelium-dependent vasorelaxations (i.e., improved this important function of the endothelium) in aortic rings exposed to 200 μM OCl^-. Based on this functional improvement, we propose that the activation of the OCl^- (and other ROS)–DNA injury–PARP pathway play a role in the pathogenesis of OCl^--induced endothelial dysfunction, which is also supported by our immunohistochemical results with PARP inhibition (prevention of OCl^--induced PARP activation).

Presumably by indirect mechanisms (discussed above), OCl^- triggers the induction of DNA strand breaks (as indicated also by our TUNEL labeling) that are the obligatory triggers of activation of PARP, which mediates the cellular response to DNA injury (11). As shown by several previous studies, the excessive PAR formation (as evidenced in our experiments with PAR immunohistochemistry) results in a cellular energetic crisis (rapid depletion of intracellular NADPH and ATP pools), which subsequently causes impaired eNOS activity and the reduced ability of endothelial cells to produce NO when stimulated by an endothelium-dependent relaxant agonist, such as ACh (40,

41). Pharmacologic inhibition of PARP can effectively prevent the energy-depleting ADP-ribose polymerization (as verified by decreased PAR formation in our present study), and by preserving NADPH and ATP pools it may protect endothelial function. Supporting this concept, previous studies have reported that PARP inhibition also prevents the endothelium-dependent relaxant dysfunction in vascular rings exposed to other reactive species, peroxynitrite (42), and hydrogen peroxide (43). However, obvious evidence for the proposed mechanism could be provided by directly measuring tissue NADPH and ATP levels and eNOS activity, but this was not conducted in the present study, and therefore, alternative explanations for the current findings are also theoretically possible.

As expected, PARP inhibition did not affect OCl^--induced immunoreactivity for NT and HNE, since they are markers of effects of OCl^- upstream from activation of the PARP enzyme (discussed above).

Results of our additional experiments in the INO-1001 control group showed that INO-1001 did not affect vascular function of control aortic rings in the dose used. Thus, the improved endothelial function seen in the OCl^- + INO-1001 treatment group is a specific phenomenon (i.e., preservation of the endothelial responsiveness) rather than the consequence of some nonspecific direct vascular effects of INO-1001.

In conclusion, the present study investigated the oxidative injury and impairment of endothelium-dependent relaxant responsiveness induced by OCl^- in rat aorta. We explored the pathophysiologic role of the OCl^-–DNA injury–PARP pathway in this impairment by demonstrating favorable effects of pharmacologic PARP inhibition on OCl^--induced, endothelium-dependent vasorelaxation. The current data further support the notion that PARP inhibition may represent a potential therapy approach to reduce vascular dysfunction induced by oxidative stress.

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