Protective Effect of Nitric Oxide on Isolated Rat Hepatocytes Submitted to an Oxidative Stress

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We have previously suggested that the nitric oxide-cyclic guanosine monophosphate (NO-cGMP) pathway protects both hepatocytes and endothelial cells against liver ischemia-reperfusion injury in rat. We study here the ability of NO to protect isolated hepatocytes against an in vitro oxidative stress induced with hypochlorite solution (ClO^-). The severity of ClO^--induced stress was quantified by the measurement of total glutathione and membrane lipid peroxidation. Cell damage was assessed by morphologic (cell viability and bleb formation) and biologic (transaminase release) criteria. A 30-minute incubation of hepatocytes with 100 μmol/L ClO^- maximally decreased cell viability (-40%) and increased bleb formation (+300%) and release of transaminases activities (aspartate transaminase [AST] = +60% and alanine transaminase [ALT] = +300%). A good correlation was observed between morphologic and biologic criteria. A preincubation of cells with 50 μmol/L 8-Br-cGMP, did not affect the adverse ClO^- effects on the morphologic criteria. In the presence of 20 μmol/L spermineNONOate, an NO donor, ClO^- did not decrease cell viability, whereas its deleterious effects on bleb formation was unchanged. A preincubation with a specific inhibitor of the soluble guanylate cyclase, the 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ; 1 μmol/L), did not affect the beneficial effect of NO on the cell viability. Our results suggest that NO protects hepatocytes against oxidative stress by a mechanism, which is cGMP-independent. However, taking into account the cytoprotective effects of cGMP in the liver, it is likely that the rapid effect of NO observed in vitro is relayed in vivo by a more long-lasting mechanism, which would be inhibited by ODQ and mimicked by 8-Br-cGMP.

PREVIOUS STUDIES HAVE demonstrated hepatoprotective effects of endogenous nitric oxide (NO) during liver ischemia-reperfusion in rat.[1,2] We have recently reported that this beneficial effect is due in part, to cyclic guanosine monophosphate (cGMP) production by soluble guanylate cyclase (sGC) activated by NO and, in part, to the antiradical properties of NO,[3] and we have suggested that the NO-cGMP pathway protects both hepatocytes and endothelial cells against ischemia-reperfusion injury.[2]

At the cellular level, the protective properties of NO are possibly due, at least in part, to its microcirculatory effects and to interrelations between the liver sinusoidal cells (endothelial and Küpffer cells) and the hepatocytes. We have suggested the involvement of a constitutive endothelial nitric oxide synthase (NOS III) in these in vivo effects.[3] A recently published report supports this hypothesis.[4]

The aim of the present study was to investigate, in vitro, the mechanism responsible for the protective action of NO against the cell damage following oxidative stress in hepatocytes during postischemia reperfusion. We have used the model described by Pella,[5] in which a suspension of freshly isolated hepatocytes is submitted to a chemical oxidative stress using hypochlorite solution (ClO^-). The severity of the ClO^--induced oxidative stress has been quantified by the measurement of total glutathione and membrane lipid peroxidation in the cell culture medium. Cell damage caused by ClO^- was assessed by morphologic and biologic criteria. In this model, we have evaluated the putative protective effects of a NO donor molecule, spermineNONOate (Sper), in the presence or absence of a selective inhibitor of sGC, and of a synthetic analogue of cGMP, 8-Bromo-cyclicGMP (8-Br-cGMP).

Our results demonstrate that NO exerts protective effects against oxidative injury in isolated hepatocytes, and that these effects are cGMP-independent.

MATERIALS AND METHODS

Hepatocytes Isolation

Isolated hepatocytes were prepared from male Sprague Dawley rats (225 to 250 g) by sequential Ca^{2+} chelation and enzymatic treatment as described by Seglen[6] with minor modifications.[7] Cells were resuspended in Williams medium (Sigma, St Louis, MO) containing 3% dialyzed bovine albumin (suspension buffer). Cells, at a concentration of about 5×10^6 cells/mL, were preincubated at 37°C in a large beaker with gentle shaking under O_2:CO_2 (95:5) atmosphere for 30 minutes. After centrifugation (1,000×*g* for 20 seconds), the supernatant and the top of the pellet (damaged cells) were removed. Cells were resuspended in suspension buffer at a concentration of approximately 5×10^6 cells/mL. Cells were counted in a hemocytometer. The viability of the cell suspension, as estimated by the Trypan-blue exclusion method, ranged between 85% and 95%.

The hepatocyte suspensions were preincubated with different molecules or with their solvents (controls) for 15 minutes before incubation with 100 μmol/L NaClO (Merck, Darmstadt, Germany): (1) 50 μmol/L 8-Br-cGMP (Sigma); (2) 20 μmol/L SpermineNONOate (Tocris-Cookson); and (3) 1 μmol/L 1*H*-[1,2,4]oxodiazolo[4,3-*a*]quinoxalin-1-one (ODQ) (Tocris-Cookson, St Louis, MO).

Effects of Oxidative Stress on Isolated Hepatocytes

ClO^- effects were estimated according to: (1) morphologic criteria, ie, evaluation of cell viability and bleb formation; (2) biological criteria, ie, activity of the enzymes aspartate transaminase (AST) and alanine transaminase (ALT) released into the culture medium, determined at 37°C with a Beckman CX 4 CE analyzer (Beckman Instruments, Villepinte, France) by using the Bio-Merieux reagent (Cayman Chemical, Ann Arbor, MI). The effects of ClO^- were expressed as a

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percentage of values measured in control cells incubated in the absence of ClO^-.

Measurement of Markers of Oxidative Stress

Measurement of reduced and oxidized glutathione (GSH+GSSG) was performed according to the method of Tietze.[8] Briefly 25 μL of cell pellet was resuspended in 100 μL Williams medium and 100 μL sulfosalicylic acid 5%, and then homogeneized. After centrifugation, the supernatant was incubated for 10 minutes with 0.43 mmol/L reduced nicotinamide adenine dinucleotide phosphate (NADPH) and 60 mmol/L 5,5-dithiobis-nitro-2-benzoic acid (Ellman reagent). Two units of GSSG reductase was then added, and the absorbance variation was read at 412 nm with a spectrophotometer (Safas, Monaco).

The malone dialdehyde (MDA) assay was performed by the evaluation of the production of thiobarbituric reactive species (TBARS) in the cell culture medium. A total of 500 μL cell homogenate was incubated for 30 minutes at 37°C with 20 μmol/L $FeSO_4$ and 2 mmol/L cysteine. After a 15-minute centrifugation at 3,200 $\times$ *g*, the supernatant was mixed 1/1 (vol/vol) with 0.60% (wt/vol) thiobarbituric acid and incubated for 15 minutes at 100°C. The absorbance was read at 532 nm with a spectrophotometer (Safas). The standard curve was established with a 10^{-2} mol/L tetrahydropropane solution in sulfuric acid.

Cell Protein Assay and Statistical Analysis

Cell proteins were determined using the Bradford method[9] with Coomassie blue. A 1-way analysis of variance was performed on logit-transformed data followed by multiple pair-wise comparisons with the Bonferroni adjustment for 5 to 7 comparisons. The overall risk was fixed at .05.

RESULTS

Cellular Effects of Hypochlorite (ClO^-)

The time course of the effects of hypochlorite on isolated hepatocytes is shown in Fig 1A and B. Incubation for 30 minutes with 100 μmol/L ClO^- maximally decreased cell viability by about 40% and increased bleb formation (+300%), the release of AST activity (+60%), and the release of ALT activity (+300%). The effects of increasing concentrations of ClO^- on isolated hepatocytes are shown in Fig 1C and D. At least up to 100 μmol/L, the effects of ClO^- on cell viability and cell enzyme release were concentration-dependent, whereas bleb formation was maximal for 10 μmol/L ClO^-. In view of these results, in the subsequent experiments we have used 100 μmol/L ClO^- to obtain more clear-cut and reproducible effects. Moreover, because in these first experiments we observed a good correlation between morphologic and biologic criteria, we then evaluated cell damage using morphologic parameters only. Thus, with 13 different hepatocyte preparations, we observed that a 30-minute incubation with ClO^- induced a 21% $\pm$ 3% decrease in cell viability ($P < .01$) and a 150% $\pm$ 23% increase in bleb formation ($P < .01$).

Effects of the 8-Br-cGMP on ClO^--Treated Hepatocytes

8-Br-cGMP was used at a concentration of 50 μmol/L. This concentration was calculated from our previous in vivo experiments[3] in which we used 16 mg/kg intravenous (IV), taking into account the extracellular compartment. This in vivo treatment efficiently protects the liver against ischemia-reperfusion

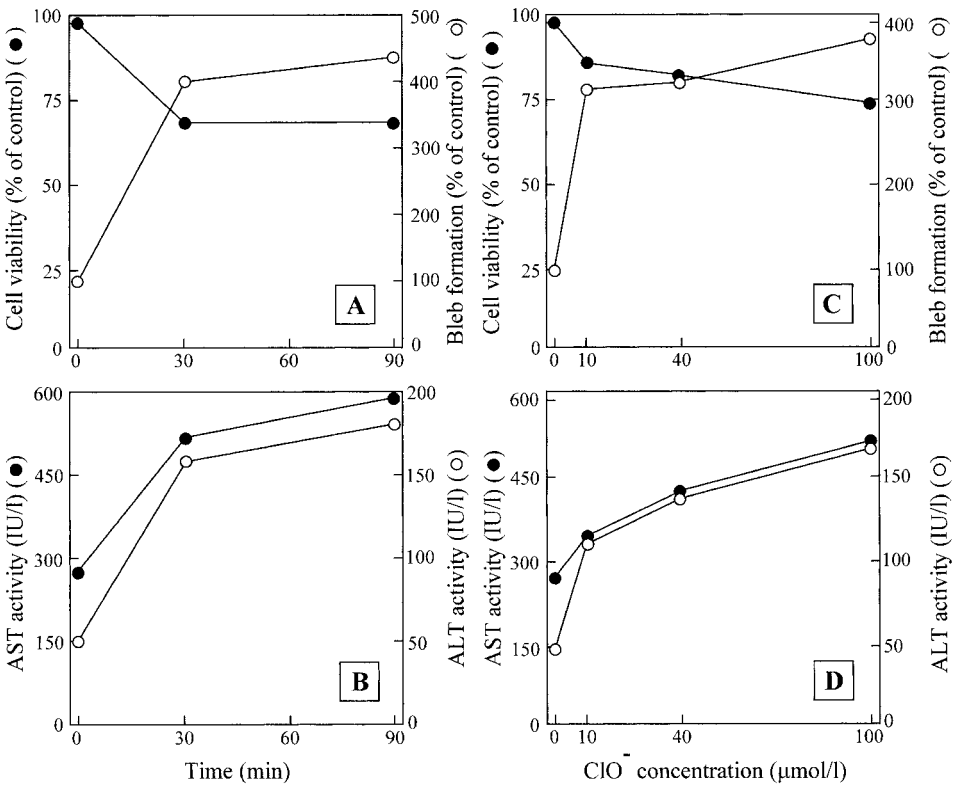

Fig 1. Dose-effect and time-effect relationships of ClO^- on cell viability and bleb formation (A and C) and AST and ALT activities (B and D) in isolated hepatocyte preparations. ClO^- effects on cell viability and bleb formation are expressed as a percentage of values for control cells. Enzyme activities are expressed as IU/L. This figure shows results of 1 representative experiment.

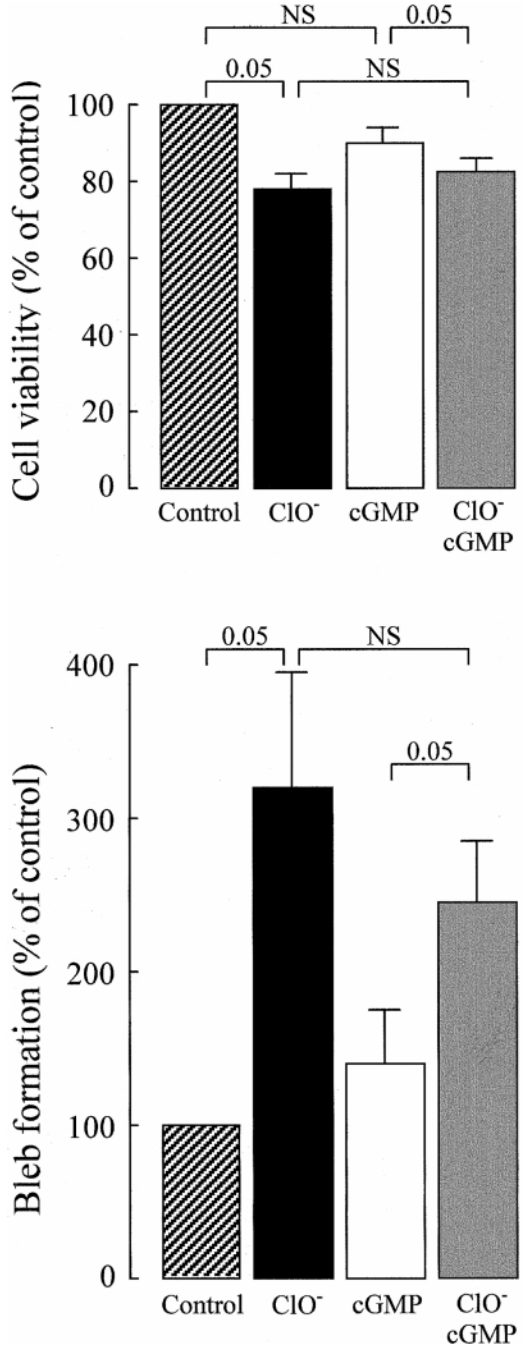

Fig 2. Effects of 8-Br-cGMP on isolated hepatocytes treated with ClO^-. cGMP effects were evaluated on cell viability and bleb formation and are expressed as a percentage of values for control cells. Cells were preincubated with 8-Br-cGMP (50 μmol/L) or its solvent for 15 minutes, ClO^- (100 μmol/L) was then added, and the incubation was continued for 30 minutes. Results are mean ± SEM of 5 different experiments. Statistical analysis was performed as indicated in Materials and Methods.

injury. Figure 2 shows that cGMP alone or in the presence of ClO^- did not affect cell viability or bleb formation. In 5 control experiments, the value of the total glutathione pool was 16.1 ± 0.9 mmol/mg of protein, a level similar to those previously reported.[10] Hypochlorite significantly decreased the total glutathione pool to 11.0 ± 1.2 mmol/mg of protein ($P < .05$) and increased MDA from 4.1 ± 0.4 μmol/mg to 5.3 ± 1.0 μmol/mg of protein ($P < .05$). In the same experiment, cGMP alone did not affect these levels and was devoid of any significant protective effect in the presence of ClO^- either on the total glutathione pool or on the MDA level (results not shown).

NO Donor Effects on ClO^--Treated Hepatocytes

The putative protective effect of NO against oxidative injury was evaluated using 20 μmol/L spermineNONOate. The choice of this concentration was based on our previous in vivo experiments, which have shown that a pretreatment with an IV injection of 5 mg/kg spermineNONOate in rats, protects the liver against ischemia-reperfusion injury (unpublished results). The calculated extracellular concentration of the NO donor corresponded to about 20 μmol/L just after injection. As shown in Fig 3, spermineNONOate alone did not affect the morphology of control cells. In the presence of the NO donor, ClO^- did not decrease cell viability, but its deleterious effect on bleb formation was unchanged. Moreover, the NO donor did not affect the modifications induced by ClO^- treatment either in the total glutathione pool or in the MDA level (data not shown).

To dissociate the direct protective effects of NO and those induced via sGC activation, we have used ODQ to inhibit the sGC. The ODQ concentration was 1 μmol/L, a dose that is able to inhibit by about 80% cGMP accumulation in response to the S-nitrosoacetylpenicillamine in an ex vivo experiment.[11] As shown in Fig 3, ODQ did not affect the beneficial effects of NO on the cell viability ($P < .05$ *v* ClO^- alone) and was without any significant effect on bleb formation. The NO donor, in the presence or absence of ODQ, was devoid of any significant effect either on glutathione pool or on the MDA level (results not shown).

DISCUSSION

A 30-minute in vitro incubation of a suspension of freshly isolated hepatocytes with 100 μmol/L ClO^- led to a 3-fold increase in bleb formation, a 20% decrease in cell viability, and a 100% to 120% increase in the release of transaminases (AST and ALT) into the culture medium. The kinetic study of all of these parameters, up to 90 minutes of incubation, showed a close parallelism between the morphologic and enzymatic criteria, with a maximal effect as early as 30 minutes. The dose-response curve of ClO^- on bleb formation was comparable to that described by Pella[5] in similar conditions, but we have extended the quantification of the effects of oxidative stress to include cell viability and the release of transaminases.

The MDA assay showed that ClO^- induced oxidative stress in the hepatocytes, although the TBARS method used underestimates the level of lipid peroxidation in isolated cells.[12] This stress was considerable, because the intracellular level of total glutathione decreased in the isolated hepatocytes. This decrease probably reflects a saturation of the glutathione reduction system[13]: the GSSG reductase activity is insufficient, and the GSSG is actively transported to the extracellular medium to preserve the redox status of the cell.[14]

The present results show that spermineNONOate exerted a

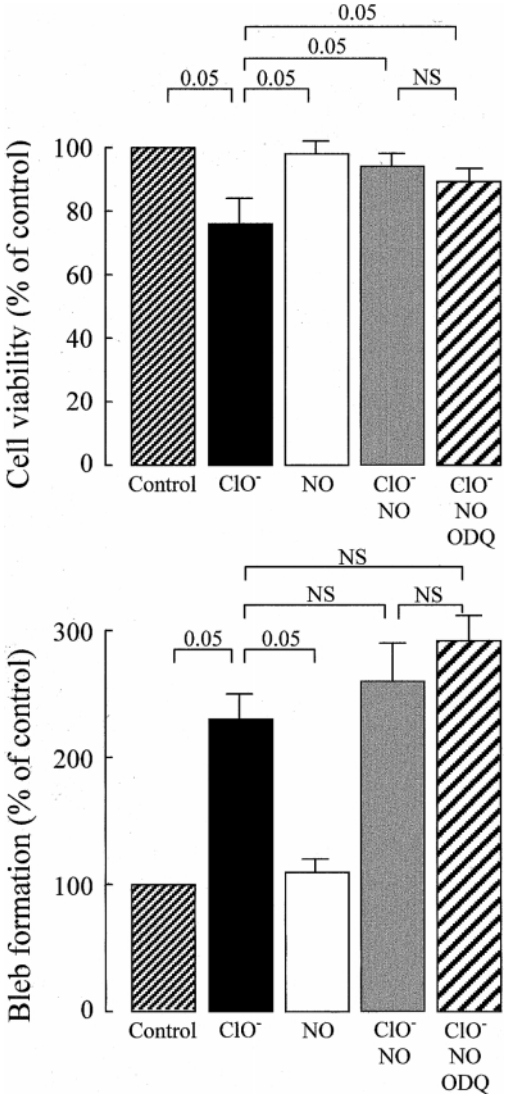

Fig 3. Effects of SpermineNONOate (NO) on isolated hepatocytes treated with ClO^- in the presence or absence of ODQ. SpermineNONOate effects were evaluated on cell viability and bleb formation and are expressed as a percentage of values for control cells. Cells were preincubated for 15 minutes with SpermineNONOate (20 μmol/L) in the presence or absence of ODQ (1 μmol/L) or solvent, ClO^- (100 μmol/L) was then added, and the incubation was continued for 30 minutes. In the presence of ODQ only, cell viability was 98% ± 5% of control and bleb formation was 110% ± 10% of control. Results are mean ± SEM of 5 different experiments. Statistical analysis was performed as indicated in Materials and Methods.

protective effect on isolated hepatocytes against ClO^--mediated oxidative stress (Fig 3), probably via the release of NO into the incubation medium. In vivo, we have previously suggested that one of the mechanisms by which NO protects against damage induced by liver ischemia-reperfusion involves, at least in part, the cGMP pathway.[3] The present data obtained in vitro demonstrated that, in our experimental conditions, 8-Br-cGMP, a cGMP analogue, did not present any protective effects either on cell viability and bleb formation or on the pool of total glutathione in hepatocytes submitted to an oxidative stress induced by ClO^- (Fig 2).

Moreover, the NO protective effects were not suppressed by a sGC inhibitor, ODQ (Fig 3). Therefore, the mechanism of NO protection appears not to involve the cGMP route. One probable explanation is that NO exerts a direct cytoprotective effect. Indeed, NO, when released at a low level during a long period as is the case with Sper, could directly protect against hepatocyte death induced by cytokines, via the S-nitrosylation of procaspases and/or the active caspase enzymes, as demonstrated in vitro[15] and in vivo.[16] The discrepancy between the results in vivo and in vitro, concerning the protective effects of cGMP, was not surprising. Indeed, the in vivo protective effect of cGMP was observed after 1 hour of reperfusion consecutive to a 45-minute period of ischemia.[3] As this cytoprotection was obtained by an IV pretreatment 30 minutes before ischemia, the protective action started at least 2 hours after 8-Br-cGMP injection. This delay should be sufficient to increase cGMP-dependent protein kinase activity (PKG) and to induce the synthesis of the stress proteins such as hsp70[17] and hsp 32 (inducible heme oxygenase-1)[18] known to exert antioxidative properties.[19,20] Conversely, in vitro, protein synthesis would not be induced during the short incubation time. Li and Billiar[21] have demonstrated a protective effect of 800 μmol/L 8-Br-cGMP against apoptosis induced by incubation with tumor necrosis factor (TNF)-α for 8 to 12 hours.

As shown in Fig 3, the NO donor alone (whose half live is about 40 minutes at 37°C at pH 7.0), did not exert, in our conditions, any cytotoxic effects at the 20 μmol/L concentration used. A preincubation of the hepatocyte suspension with Sper, 15 minutes before ClO^- addition, completely protected the cells in terms of viability, as evaluated by Trypan blue exclusion, but had no beneficial effect on bleb formation. This discrepancy between the improvement of viability and the persistence of bleb formation may be explained by the mechanism of the formation of blebs and their evolution towards cell membrane disruption. Indeed, before reaching the step of membrane lysis, evaginations can be resorbed and therefore the phenomenon is reversible.[22] We hypothesize that NO did not interfere with the formation of evaginations, but could reverse their evolution, and thus directly protect against cell death. At variance with the present data, Pella,[5] using the sodium nitroprussiate (SNP) as an NO donor, observed complete protection against bleb formation due to 1 μmol/L ClO^-. Two facts could explain this discrepancy: (1) the lower concentration of ClO^- used by these investigators and (2) the greater speed of NO production with SNP than with Sper.

In terms of cell redox status, Sper did not reduce the magnitude of the oxidative stress, because it did not significantly prevent the decrease in total glutathione induced by ClO^-, whereas it prevented the deleterious effects of oxidative stress on hepatocyte survival.

Our results suggest that, in isolated hepatocytes, NO exerts protective effects against oxidative stress by a mechanism, which is cGMP-independent. However, taking into account the cytoprotective effects of cGMP in the liver in vivo, it is likely that the rapid effect of NO observed in vitro is relayed in vivo

by a more long lasting mechanism, which is inhibited by ODQ and mimicked by 8-Br-cGMP, and which probably involves the synthesis of heme oxygenase-1.

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