Protective effect of nitric oxide on isolated rat hepatocytes submitted to an oxidative stress

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Abstract

Nitric oxide (NO) is a free radical, present in usual conditions such as an atmospheric pollutant. In the biological systems, NO has a short half-life in the order of seconds. NO has an intermediate oxidation state and is consequently able both to oxidize and to reduce the chemical compounds with which it comes into contact. The aim of the present research was to study the ability of NO to protect isolated hepatocytes against an oxidative stress "in vitro" induced by paracetamol. For the experiments, 70-80 day-old Wistar Male Rats have been used and fed with standard diet (Morini, S. Polo d' Enza, ReggioEmilia), in standard stabulation conditions. The liver cells were isolated with the "two steps" perfusion technique by Seglen (1976). The cell viability was assessed by means of the MTT test (Mosmann, 1983) and the urea production was determined by means of the colorimetric enzymatic method Urea Color 2 (Sclavo Diagnostics, Siena, Italy). The hepatocytes were treated either for 2 hours with paracetamol 40 mM only or with paracetamol 40 mM plus NOR-3 0.01 and 0.1 mM, an NO donor. After 2 hours of paracetamol treatment and 24 hours after the end of the same treatment a reduction of cell viability and urea synthesis were observed. After 2 hours of co-treatment with NOR-3 0.01 mM and paracetamol 40 mM, a reduction of cell damage was observed, while with the paracetamol alone, there are more evident metabolic damages. On the contrary the treatment with NOR-3 0.1 mM and paracetamol 40 mM did not produce any protective effect. This could confirm the double phase, dose dependent, NO effect.
The protective effects of NOR-3 at low concentrations from paracetamol damages could be due to the NO inhibitory effect on the activity of the cytochrome P-450 (Vuppugalla et al., 2004).

Introduction

Nitric oxide (NO) is a free radical, present in standard conditions such as in atmospheric pollutants and in cigarette smoke. In the biological systems NO has a short half-life in the order of seconds. NO is in an oxidation intermediate state and is consequently able to oxidize and to reduce the chemical compounds with which it comes into contact. It's biosynthesis mainly happens through the transformation of the L-arginine amino acid in citrulline. This reaction requires the participation of NO synthase (NOS) enzyme. The mechanisms by which NO can elicit changes in the hepatic metabolism can be divided into two areas by 1) exerting direct effect on hepatic uptake, storage, detoxification and clearance mechanisms and 2) exerting an indirect effect due to induction of changes in the hepatic vascular tone, which would ultimately affect these mechanisms. Currently, comparatively little is known about the direct mechanisms, and this offers a very exciting area of research (Barry, 1998).
In the present study we have investigated "in vitro" the action of NO against the cell damage following oxidative stress in hepatocytes treated with paracetamol.
We have evaluated the effects of the NO-donor NOR-3, that spontaneously release NO at physiological pH (Yamamoto et al., 2000), on isolated rat hepatocytes submitted to paracetamol oxidative stress. Paracetamol, a widely used analgesic and antipyretic, results in severe hepatic centrilobular necrosis when taken in excess of therapeutic doses (Cohen et al., 1998). This is attributed to its activation by cytochrome P-450 to a highly reactive metabolite, N-acetyl-p-benzoquinone-imine (NAPQI), that is a strong electrophile and a potent oxidizing agent (Jaeschke et al., 2003).
We have investigated cell viability and urea biosynthesis by using spectrofluorimetric and spectrophotometric techniques.

Materials and Methods

Hepatocytes were isolated from male rats, Wistar strain (180 to 200 gbw) stabulated in standard conditions, by a modification of the method of Seglen (1976). All procedures on the animals were performed according to the CEE directive n. 86/609 on animal experimentation. The viability of the cells was higher than 80%, as estimated by trypan blue dye exclusion test (Kaltenbach et al., 1958). After cell counting the cells were diluted at a concentration of 5×10^5 cells/ml with incomplete medium supplemented with 2% fetal calf serum, 0.1U/ml insulin and 10^{-6} M dexamethasone (complete medium). The hepatocytes were then plated in 24 well-plates coated with rat tail collagen at the final cell density of 2.5×10^5 cells per well and incubated at 37°C in an humidified atmosphere of 5% CO_2 and 95% air. After 6 hours incubation, the medium was changed and replaced with incomplete medium to remove dead cells.
After other 24 hours of culture, the hepatocytes are exposed to the substances to be tested.
In order to determine the NO levels, the cells were incubated for 120 minutes with the probe DAF-2DA (10 μM) (Kojima et al., 1998). Then, the reading of the plate in fluorescence ($\lambda_{ecc.}$=495 nm; $\lambda_{em.}$=515 nm) is carried out.
Urea levels in the medium were measured by spectrophotometric method using Urea Color 2 Kit (Sclavo Diagnostics, Siena, Italia) measuring absorbance at 600 nm. Urea synthesis was calculated as ng urea per cell per hour.
Cell viability was determined by MTT test method (Mosmann, 1983). For the determination of cell viability, culture medium has been discarded and MTT solution was added and incubated for 3 hours. At the end of the incubation period the MTT solution was removed and the cells and dye crystals were dissolved by adding dimethylsulfoxide (DMSO). Absorbance was measured at 570 nm in a Shimadzu UV-2100 Spectrophotometer and the results were expressed as a percentage of the absorbance of the samples in comparison to control.

Statistical analysis

At least four independent determinations of each parameter were compared to control using Student's T-test. Differences were considered significant when $p < 0.05$ was obtained.

Results and discussion

In order to study the effects of nitric oxide, on the cell viability and on the urea biosynthesis in isolated hepatic cells submitted to an oxidative stress, we used the NO-donor, NOR-3. The NO-donors are a family of substances that spontaneously releases NO at physiological pH. The NOR-3 has a $t_{1/2}$ of 105 minutes.
The hepatocytes were treated either for 2 hours with paracetamol 40 mM (Lewerenz et al., 2003) only or with

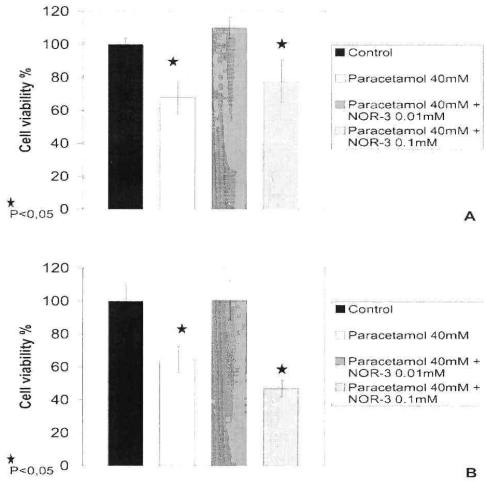

Fig. 1 - *Determination of cell viability in hepatocytes treated with 40 mM paracetamol only or with paracetamol 40 mM plus NOR-3 0.01 and 0.1 mM. Cell viabilility was spectrophotometrically determined at 570 nm by MTT assay in hepatocytes incubated in basal conditions (black column), in presence of 40mM paracetamol (white column), in presence of 40mM paracetamol + 0.01mM NOR-3 (hatched column) and in presence of 40mM paracetamol + 0.1mM NOR-3 (crosshatched column) for 2 h period. (A) Cell viability determined immediately after. (B) Cell viability determined after an additional 24 h incubation period. Results are expressed as a percentage of control. Values are the means ± S.E.M. (bars) of four independent experiments. *P<0.05 compared with control.*

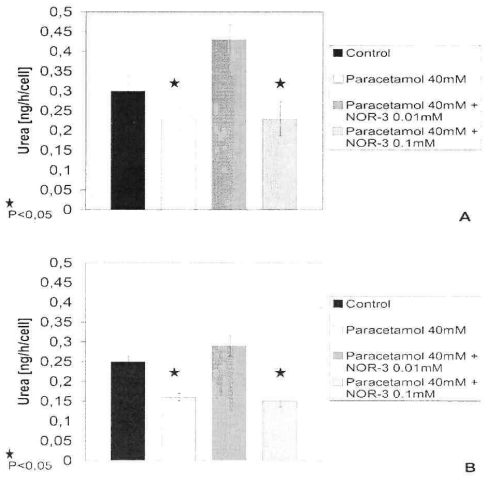

Fig. 2 - *Determination of urea production in hepatocytes treated with 40mM paracetamol only or with paracetamol 40 mM plus NOR-3 0.01 and 0.1 mM. Urea production was spectrophotometrically determined at 600 nm after 2 h incubation in basal conditions (black column), in presence of 40 mM paracetamol (white column), in presence of 40mM paracetamol + 0.01 mM NOR-3 (hatched column) and in presence of 40mM paracetamol + 0.1 mM NOR-3 (crosshatched column). (A) Urea production determined immediately after. (B) Urea production determined after an additional 24 h incubation period. Values, expressed as ng urea per cell per hour, are the means ± S.E.M. (bars) of four independent experiments. *P<0.05 compared with control.*

paracetamol 40 mM plus NOR-3 0.01 and 0.1 mM. After 2 hours of paracetamol treatment and 24 hours after the end of the same treatment a reduction of cell viability was observed; after 2 hours of co-treatment with NOR-3 0.01 mM and paracetamol 40 mM, a reduction of cell damage was observed, on the contrary the treatment with NOR-3 0.1 mM and paracetamol 40 mM did not produce any protective effect (Fig 1A-B). The same course has been observed for the biosynthesis of the urea (Fig. 2A-B). This could confirm the double phase, dose dependent, NO effect.

The protective effects of NOR-3 at low concentrations from paracetamol damages could be due to the NO inhibitory effect on the activity of the cytochrome P-450. The inhibition of the cytochrome P-450 could reduce the oxidative damage caused by the metabolism of paracetamol (Vuppugalla et al., 2004).

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