Peroxynitrite generated from constitutive nitric oxide synthase mediates the early biochemical injury in short-term cultured hepatocytes

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Abstract Early loss of P450 in rat hepatocyte cultures appears directly related to nitric oxide (NO) overproduction. This study provides experimental evidence for the induction – shortly after isolation through the classical procedure – of strong oxidative stress that involves both oxygen-derived and NO-derived species. NO formation at this stage is due to the early activation of liver constitutive NO synthase (cNOS). Immunodetection of nitrated proteins provides direct evidence of endogenous peroxynitrite (PN) formation upon hepatocyte isolation. On the basis of the combined use of dihydrorhodamine 123 and NOS inhibitors, the analysis of the amount, time course and nature of the species involved supports the view that PN generated from cNOS-derived NO, while not affecting cell viability and hepatocyte monolayer development, is the main species likely responsible for the early biochemical injury commonly observed in hepatocyte cultures.

Key words: Hepatocyte isolation; Culture; P450 content; Nitric oxide synthase; Oxidative stress; Peroxynitrite; Protein nitration

1. Introduction

The biological chemistry of nitric oxide (NO) in the oxygenated cellular environment is extremely complex: it involves the direct interaction of the NO radical with specific biomolecules and the so-called indirect effects [1], due to secondary more potent oxidant species (reactive nitrogen species, RNS) derived from NO autoxidation which are able to react with DNA, lipids, thiols and transition metals [1,2]. In addition to its regulatory role as a signalling molecule [2–5] it has become evident that NO (or NO-derived species) is a critical factor involved in various toxicological mechanisms [1,6–8]. Some controversy exists however about the damaging vs. protective actions of NO on oxidative injury, whose biological significance in living cells and tissues remains ill-defined.

Research in this laboratory [9] has recently shown that NO synthesis is activated in hepatocytes from control rats during cell isolation through the conventional collagenase-based procedure, thus providing experimental evidence that endogenous NO is responsible for the early impairment of mitochondrial function which leads to transient cell energy depletion and significant changes in hepatocyte glucose metabolism as previously reported [10]. Results also showed that spontaneous NO overproduction is directly related to quick and irreversible loss of P450 content in rat hepatocyte primary culture [9]. Hence, this study was conducted to further characterize the operative pathway responsible for NO synthesis in freshly isolated hepatocytes and the radical mechanism(s) underlying the generalized loss of specific liver functions in short-term cultures. The current work demonstrates that NO generation at this stage is due to the early activation of the liver constitutive Ca^{2+}-dependent nitric oxide synthase (cNOS). The results provide first evidence for the existence of a significant intracellular oxidative stress upon hepatocyte isolation which involves both reactive oxygen species (ROS) and RNS derived from NO. Peroxynitrite[1] (PN) formation is evidenced by the detection of protein-bound 3-nitrotyrosine. The analysis of the time course of reactive species formation and the use of inhibitors of cNOS activity supports the view that PN – without affecting cell viability – is the main species involved in hepatocyte metabolic impairment.

2. Materials and methods

2.1. Cell isolation and culture

Hepatocytes isolated from fed male Sprague–Dawley rats (180–250 g) by reversed liver perfusion with collagenase (Boehringer Mannheim) were cultured on fibronectin-coated dishes at 37°C under a 5% CO_2 atmosphere [10]. The duration of the whole procedure never exceeded 30 min. Cells were cultured in phenol red-free Ham's F12 medium plus antibiotics, 0.2% bovine serum albumin, 10^{-8} M insulin and 2% calf serum (Gibco). Unattached cells were removed by renewing the medium 1 h after plating. Viability was assessed by the trypan blue dye exclusion test and always exceeded 90%. N^G-Nitro-L-arginine methyl ester (L-NAME, Sigma) and dihydrorhodamine 123 (DHR 123, Sigma) were added as indicated.

2.2. Electrophoresis and Western blot analysis of NOS

At indicated times, isolated cells or monolayers were frozen under liquid N_2 and kept at −80°C. Whole cell extracts (5–10 mg protein/ml) were prepared by brief sonication on ice-cold 50 mM Tris–HCl buffer (pH 7.4) plus protease inhibitors (2 μM leupeptin, 1 μM pepstatin, 1 μM aprotinin, 1 mM PMSF and 0.5 mM aurintricarboxylic acid). Microsomal and cytosolic extracts were obtained by centrifugation (100 000 × *g*, 90 min) of the S9 fraction from each crude homogenate. Membrane pellets were homogenized in the same buffer plus

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Abbreviations: NO, nitric oxide; iNOS, inducible nitric oxide synthase; cNOS, constitutive nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methyl ester; DHR 123, dihydrorhodamine 123; PN, peroxynitrite; RH, rhodamine; ROS, reactive oxygen species; RNS, reactive nitrogen species derived from NO

[1] The term peroxynitrite is used to refer to peroxynitrite-derived intermediates (i.e. peroxynitrite anion, peroxynitrous acid and/or reactive species formed from the reaction of peroxynitrite with carbon dioxide).

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20% glycerol. Samples were kept at −20°C until processed. Proteins (10 μg/well) were resolved (SDS–PAGE, 4–15% gradient gels) and transferred (SD-transfer, Bio-Rad) to nitrocellulose. Immunodetection was performed with a rabbit polyclonal antibody to cNOS and a monoclonal anti-iNOS antibody (Transduction Laboratories) followed by incubation with their respective peroxidase-conjugated secondary antibodies. Proteins were detected using the Opti-CN system (Bio-Rad).

2.3. Formation of ROS and RNS in living hepatocytes

DHR 123 was used as a probe for ROS and RNS derived from NO [11,12], in assays here adapted to quantitate intra- and extracellular oxidation to fluorescent rhodamine (RH). To study RH formation as a function of time and culture conditions, DHR 123 (20 μM) was added at the moment of cell plating and kept present for the whole culture period. Aliquots from the medium were then taken regularly in which extracellular RH was determined. Intracellular RH accumulation was determined in parallel plates that were quickly frozen at the indicated times. For kinetic analysis of the rate of reactive species formation, cells were cultured in standard medium (±1 mM L-NAME) for the indicated times. DHR 123 (20 μM) was then added and the rate of RH generation in the medium was determined in a short (60 min) incubation period. RH was quantitated fluorometrically [13] against the appropriate standard curves. Data were corrected for autofluorescence and DHR 123 autooxidation.

2.4. Immunodetection of protein-bound 3-nitrotyrosine

Whole cell extracts were prepared as indicated above. Protein (20 μg/well) was resolved by electrophoresis on 12.5% SDS–polyacrylamide gels and transferred to nitrocellulose. Immunodetection was performed using a monoclonal anti-3-nitrotyrosine antibody (Calbiochem) followed by peroxidase-coupled chemiluminescence detection (ECL kit, Amersham). Specificity was confirmed by blocking the primary antibodies with 10 mM buffered 3-nitrotyrosine prior to membrane incubation.

2.5. Other determinations

NO_2^- and total nitrites (NO_2^-+NO_3^-) were determined fluorometrically [14]. NO_3^- formation was estimated as the difference between total nitrites and NO_2^- in the same medium aliquot. Cellular GSH content and aspartate aminotransferase leakage were determined by standard procedures [15,16]. Protein content was determined by the Lowry procedure.

2.6. Statistics

Experiments were performed in at least three different cultures with 3–4 plates per variable. Statistical analysis was done by a two-tailed Student's *t*-test. A value of $P < 0.01$ was considered significant. Otherwise representative experiments are shown.

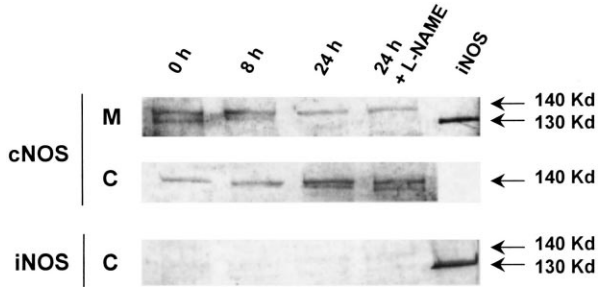

Fig. 1. Western blot analysis of cNOS and iNOS protein in cultured hepatocytes. Cytosolic (C) and microsomal (M) extracts from the same cell preparation were processed as indicated (see Section 2). iNOS: lysates from RAW 264.7 macrophages as positive controls for iNOS immunoreactivity. As shown, there is cross-reaction of the polyclonal anti-cNOS antibody to the iNOS protein while the monoclonal anti-iNOS antibody weakly recognizes hepatocyte cNOS. An identical pattern was obtained for three different cultures at several culture times.

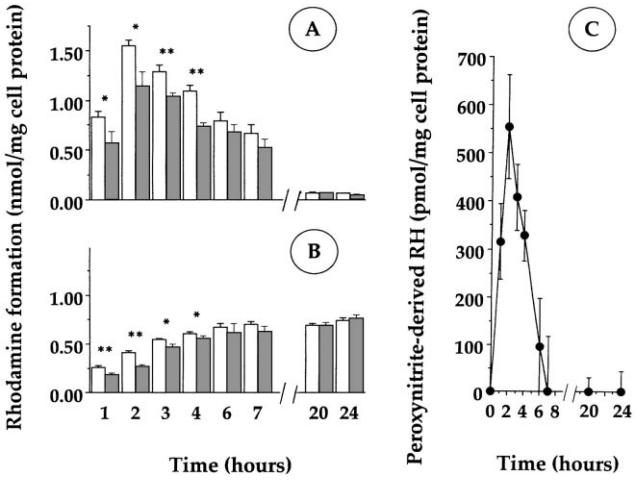

Fig. 2. Time course of RH accumulation in cells (A) and media (B). Hepatocytes were cultured in standard medium plus 20 μM DHR 123, supplemented (shaded bars) or not (clear bars) with 1 mM L-NAME. Data (normalized to mg cell protein for comparison) are the mean ± S.D. of four dishes from one representative culture. *$P < 0.01$, **$P < 0.001$. C: Estimation of total RH formation due to PN.

3. Results

3.1. cNOS as the major source of NO in short-term cultures

Fig. 1 analyzes the time course and the subcellular distribution of the two major NOS isoforms in hepatocytes following cell isolation. Western blot analysis of cytosolic extracts shows that the iNOS protein is not significantly detectable for the whole culture period in spite of the fact that, as previously shown [9], the pattern of NO_3^-+NO_2^- accumulation in the medium (an indirect measurement of NOS activity) would have better suggested induction of iNOS. In fact, cNOS is the only enzyme present in hepatocytes (Fig. 1) and the protein appears equally distributed between the microsomal and cytosolic compartments. No significant changes were observed in the apparent amount of cNOS for up to 24 h of culture nor did addition of 1 mM L-NAME to the medium, while efficiently blocking NOS activity [9], significantly modify the subcellular distribution (see 24 h+L-NAME, Fig. 1) and time course evolution (not shown) of cNOS content.

3.2. Activation of oxidative stress as a very early event upon hepatocyte isolation: differential role of ROS and RNS derived from NO

As shown (Fig. 2A,B) oxidative stress is acutely induced from the very early stages of culture: RH accumulation in cells and medium significantly increases for the first 2–4 h of culture decreasing slowly thereafter so that, from 6 to 24 h of culture no additional formation of RH is observed. Limited DHR 123 availability can be reasonably excluded since by 6 h of culture (steady-state RH levels) total DHR 123 loss (from autoxidation, cellular and intracellular oxidation) accounts for less than 2 μM. DHR 123 oxidation is known to involve different oxidant species [12]. The current view is that under oxidative stress conditions in which NO production also occurs, a role for both ROS and RNS should be considered. A straightforward approach to monitor the contribution of PN to DHR 123 oxidation is simply to inhibit NO production. In our study the time required for efficient inhibition of cNOS by

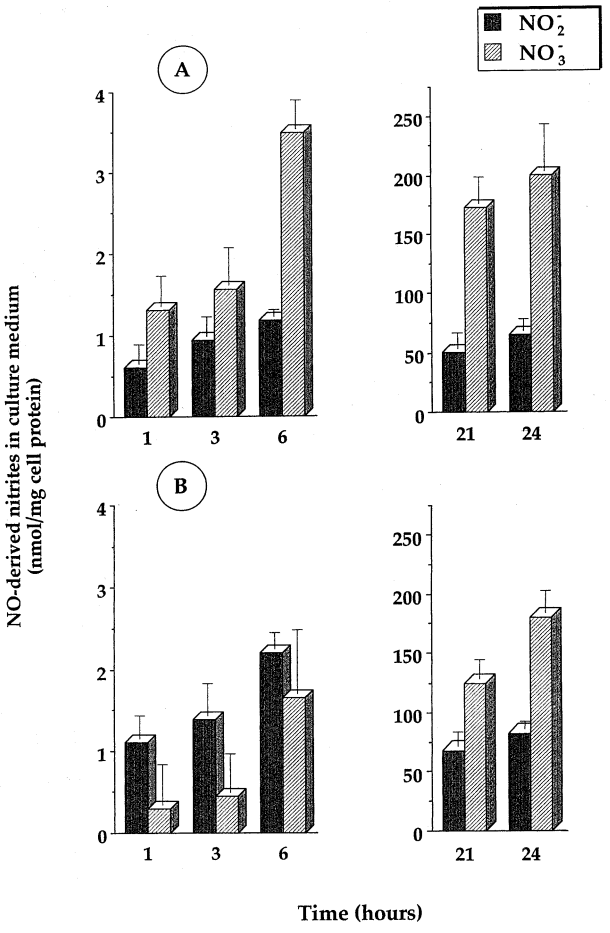

Fig. 3. NO_2^- and NO_3^- accumulation in control cultures as a function of the presence of DHR 123. Cells were isolated and cultured by the standard procedure (see Section 2) and 20 μM DHR was added (B) or not (A) at the moment of cell plating (0 h). Data (mean ± S.D. of four dishes) correspond to one representative culture.

L-NAME is about 30 min (not shown), which likely represents the minimum required for cell internalization and esterase action [17]. As shown, exposure to 1 mM L-NAME significantly reduces (by about 30%) the amount of RH accumulated in cells (Fig. 2A) and medium (Fig. 2B) for the first 4 h of culture. RH formation when NO synthesis is efficiently blocked [9] presumably results from DHR 123 reactivity towards ROS: the time course found is equivalent to that in controls which indicates that oxidative stress due to ROS is not influenced by inhibition of NO synthesis. Fig. 2C is an estimation of the total (extracellular+intracellular) PN formation in hepatocytes as derived from the corresponding RH cumulative curves. This suggests that PN formation in standard cultures is a very early event upon cell isolation, which proceeds significantly for only the first 4 h of culture. In the same time period (not shown for brevity) a significant reduction of hepatocyte GSH was observed (50% of initial by 4 h) that later recovers to initial levels.

Besides its role as a label for ROS and RNS generation in living cells, DHR 123 may also act as a 'scavenger' for both kinds of species. The ability of DHR 123 to efficiently trap PN is indicated by the lowered levels of NO_3^- recovered in the medium for the first 6 h in cultures exposed to the probe (Fig.

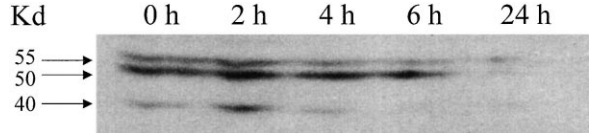

Fig. 4. Western blot detection of nitrated proteins in hepatocyte lysates as a function of time in culture. Immunodetection was performed as indicated in Section 2. Molecular weight standards (not shown) were used to approximate the size of the immunoreactive proteins. A similar pattern was obtained for three different hepatocyte cultures.

3B) as compared to controls (Fig. 3A) in which the NO_3^-/NO_2^- ratio stays constant for the whole 24 h culture period (mean value 2.7 ± 0.3). DHR 123 also diminishes oxidative stress-induced hepatocyte injury as indicated by a significant reduction in the very low basal aspartate aminotransferase leakage (not shown) in control cultures, which otherwise is not significantly influenced by L-NAME. Cell viability is not modified by exposure to either L-NAME or DHR 123, but monolayer morphology and cell protein recovery by 24 h appear clearly improved in L-NAME-exposed cultures [9].

3.3. Protein nitration in short-term cultured hepatocytes

PN and/or PN-derived intermediates can nitrate free or protein-bound tyrosine residues to form 3-nitrotyrosine. The detection of protein-bound 3-nitrotyrosine is often used as a stable and specific 'footprint' for PN generation in living cells [18]. Fig. 4 shows the pattern of nitrated proteins on hepatocytes isolated and cultured in standard conditions and thus provides direct evidence that PN is actually formed from the very early stages of liver perfusion. Endogenous tyrosine nitration is already significant at 0 h (isolated cells) and increases for the next 2 h in culture. The level of nitrated proteins decreases thereafter so that by 24 h of culture they are not longer detectable. Three major immunoreactive bands are detected (with molecular masses of 40, 50 and 55 kDa) which appear to follow an identical time course pattern.

3.4. Rate of ROS and peroxynitrite formation by hepatocytes as a function of time in culture

Specific assays were designed to determine the actual rate

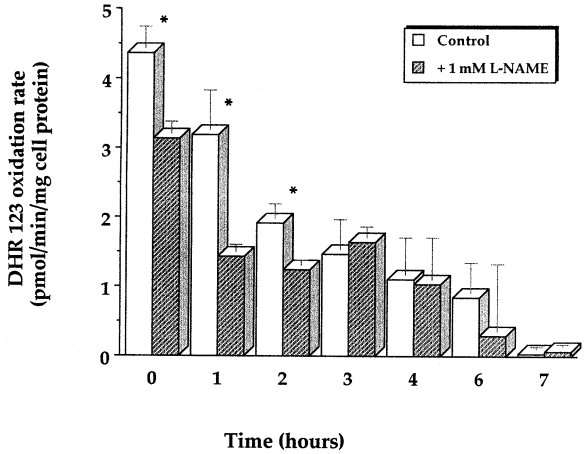

Fig. 5. Rate of extracellular DHR 123 oxidation in the presence and absence of 1 mM L-NAME. The rate of RH formation was estimated from the slopes of the kinetic assay (see Section 2). Data are the mean ± S.D. from three different cultures. *$P < 0.01$.

for oxidant species formation by hepatocytes: due to the very early culture period involved this could only be accurately achieved extracellularly. In this assay RH formation in the medium stays constant for up to 60 min (not shown). As first suggested in Fig. 2B, the rate of DHR 123 oxidation in controls (which accounts for ROS+PN) decreases progressively (Fig. 5) and is negligible by 6–7 h of culture. Kinetic analysis in cultures ±1 mM L-NAME provides an estimation of the rate of PN formation upon cell isolation and culture (Fig. 5): PN formation appears only significant for the first 2 h while oxidation due to ROS is observed for at least 4 h following hepatocyte isolation.

4. Discussion

In a previous work [9] we showed the importance of endogenous NO as a causal factor responsible for important phenotypic changes in short-term cultured hepatocytes including the well-known early and irreversible loss of P450. This study aims to further characterize the mechanism underlying this phenomenon. First, results allow us to identify cNOS (but not iNOS) as the major NOS isoform detectable for the whole initial 24 h culture period. So, the spontaneous generation of high NO amounts appears to result from the very early activation of the hepatocyte constitutive enzyme. The second finding is that hepatocytes are under strong oxidative stress that lasts for at least the first 4 h of culture. Thus, as for cNOS activation, induction of cellular oxidative stress is a very early event upon hepatocyte isolation. Results finally show that PN is generated at significant rates within this period. As discussed, this suggests a key role for PN as the main reactive species likely involved in the early biochemical injury observed in short-term hepatocyte cultures.

The analysis of the amount, the time course and the nature of the oxidative stress that actually has an impact on the hepatocyte (as both source and target cell) has been based on the combined use of DHR 123 and L-NAME, an inhibitor of NO formation particularly efficient for cNOS [17] which does not interfere with RH formation [19]. DHR 123 oxidation is utilized for the detection of both ROS (an H_2O_2-driven process via peroxidase- or metal-dependent pathways) and NO-derived intermediates (direct oxidation mainly due to PN) [11,12]. Its membrane diffusion properties and the fact that RH is selectively retained by living cells [13] made DHR 123 a useful marker in both cell and media. Intracellular oxidation of the probe (more than two-fold that detected extracellularly) is consistent with the endogenous origin of the species involved. As long as mitochondrial and plasma membrane redox potential remains intact RH is retained inside the cell, although some dye leakage cannot be excluded following long-term exposure [13]. Thus, extracellular RH accumulation – especially during the initial 2 h period – is likely to be the result of DHR 123 oxidation in the same medium from reactive species able to diffuse outside the intact hepatocyte. The possibility that once generated in the medium RH may have diffused into the cells – and thus be detected as intracellular – can be reasonably excluded from the extended linearity in the kinetic assays (60 min, not shown) and the fact that the maximal RH concentration achieved in the medium is only 0.5 μM while at least 20–30 μM RH is required for optimal mitochondrial staining [13].

One major fate of NO is its extremely fast reaction with the superoxide anion to yield PN [1], a far more reactive species than its radical precursors. Efficient suppression of NO synthesis by L-NAME provides indirect evidence that, in addition to ROS, significant amounts of PN are readily formed in short-term cultured hepatocytes. PN is mainly generated inside the cell (Fig. 2A) but the PN-derived RH accumulation in the medium (Figs. 2B and 5) suggests that it may also be partially formed extracellularly. We cannot exclude, however, that intracellularly generated PN might have also diffused across the cell membrane [20]. Time course analysis of NO-dependent oxidation of DHR 123 (intracellular+extracellular) reveals that PN is actively formed shortly after hepatocyte isolation but significantly detectable for only the first 4 h of culture (Fig. 2C). Cumulative curves also suggest that PN generation proceeds linearly for the first 2 h. Immunodetection of nitrated tyrosine residues in hepatocyte proteins adds strong support to these findings. Protein nitration depends on the concentration and on the time of exposure to PN. Results show that nitrated proteins are already present in freshly isolated hepatocytes (after 30 min of liver perfusion) but quantitatively increase for the next 2 h in culture, a time frame coincident with that estimated for PN formation in DHR 123 studies. Three major nitrated bands have been identified as preferential protein targets in hepatocytes. Interestingly, the nitrated proteins progressively disappear at later culture times, being negligible by 24 h. Recent studies on different cellular systems [21–24] point to the fact that nitration of specific proteins by PN – which can modify protein properties, structure and function – may be part of its pathological mechanism of action. However, little is known about the turnover of nitrated proteins in the living cell. The disappearance of nitrated proteins in our study may involve their own half-lives but also cell enzymatic cleavage of nitrated tyrosine residues (without altering the degradation of the whole nitrated protein per se) may play a role [24,25]. Studies aimed to characterize these proteins and their cellular turnover are now in progress.

The rate of extracellular PN-derived RH formation ranged from 1 to 1.5 pmol RH/min/mg cell protein (1 h of culture) for the different cell preparations examined in this study. Nevertheless the actual rate of PN formation in the hepatocyte is expected to be higher since (i) our calculation is based on the assumption that ROS-dependent DHR 123 oxidation will not be affected by inhibition of NO synthesis, which is not necessarily true [12], (ii) oxidation of each DHR 123 molecule requires more than two molecules of PN [11], and (iii) most PN is generated intracellularly (Fig. 2A). Also, as previously shown [9,10], lactate formation is strongly activated for the first 0–4 h of hepatocyte culture, which leads to a significant decrease in extracellular pH (from 7.40 to 6.98). Intracellular acidosis within this period (as observed in rat livers in hypoxic conditions [26]) could have favored PN protonation as well as its reaction with carbon dioxide and thus contribute to enhance PN reactivity (via peroxynitrous acid and nitrosoperoxycarbonate anion).

We have attempted to define the fate of NO synthesized by hepatocytes and its relation to the biochemical injury detected in short-term culture. Cell isolation leads to the activation of cNOS and the prompt generation of intracellular oxidant stress. As long as ROS formation is operative (0–4 h of culture), cNOS-derived NO diverts to PN generated in significant amounts from the simultaneous flux of superoxide and NO

(thus, very low levels of $NO_2^-+NO_3^-$ are detected in the medium at this stage, ca. 5 nmol/mg cell protein by 6 h of culture [9]). The bulk of PN is intracellular which ensures accessibility to its cellular target molecules [1,2]. This is further confirmed by the detection of nitrated proteins at this stage. At later phases (6–24 h of culture) ROS are no longer detectable but NO formation still proceeds actively for up to 24 h: within this period the main biological fate of NO appears the spontaneous decay to stable end-products ($NO_2^-+NO_3^-$) which accumulate in the culture medium (up to 275 nmol/mg cell protein [9]). Although a role for higher nitrogen oxides cannot be completely ruled out, results in this and our previous study [9] suggest that PN rather than NO itself is likely the ultimate mediator of quick and irreversible loss of hepatocyte specific functions. The inhibition pattern of mitochondrial function and the time course of P450 irreversible loss [9] closely resemble the time frame of PN generation in short-term cultures. In fact it may be suggested that P450, the most abundant hemoprotein in liver cells, is a very early and accessible target for this effector molecule. Furthermore, L-NAME does not modify the pattern of ROS formation (even if added at the very early moment of liver perfusion, results not shown) but the sole inhibition of NO synthesis – which precludes PN generation – is enough to completely prevent metabolic injury and P450 loss [9]. Sources for ROS in hepatocytes include mitochondrial respiration, xanthine oxidase and the uncoupled activity of both P450 and cNOS enzymes. As for the early activation of cNOS, factors leading to oxidative stress in hepatocytes appear inherent to the conventional isolation procedure (collagenase perfusion method) but remain unidentified. As suggested before [9] this may involve calcium movements, unknown contaminants of commercial collagenase (i.e. endotoxin) or peptides derived from extracellular matrix but even mechanical shear stress, ischemia–reperfusion or hyperoxia (the medium is gassed with 95% O_2/5% CO_2) due to the in situ liver perfusion procedure may play a role. Interestingly, a recent study [27] has reported induction of NF-κB binding by just 10 min of the commonly employed hepatocyte isolation method: although not evaluated, authors suggest a role for NOS in this process.

In conclusion, the procedure currently used for hepatocyte isolation actually induces endogenous oxidative stress, which involves both ROS and RNS. Oxidative stress is not linked to irreversible cellular damage, since functional monolayers are routinely obtained in conventional culture conditions. However, our study establishes that ROS formation at this stage plays an indirect key role since it allows PN formation from cNOS derived NO. PN (and possibly other intermediates derived from the NO/O_2 reaction) appears in turn the only critical factor for the irreversible phenotypic changes (i.e. early P450 loss) consistently observed in rat hepatocyte primary cultures. Prevention of NO formation through the early addition of NO biosynthesis inhibitors appears the most reasonable approach to overcome these events [9]. The existence of a low-output, Ca^{2+}-dependent cNOS in hepatocytes has been known for a long time [28] but – probably because most attention has been devoted to hepatocyte iNOS induction – its biological significance still remains obscure. Also, formation of PN in living cells has been claimed to be mainly dependent on iNOS activity [1]. This study demonstrates that significant amounts of PN can be generated in hepatocytes from cNOS-derived NO flux. The role of cNOS activity on liver metabolic modulation and its implication in different pathophysiological situations should therefore be considered.

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