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Rapid Induction of NF-κB Binding during Liver Cell Isolation and Culture: Inhibition by *L-NAME* Indicates a Role for Nitric Oxide Synthase

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This study is the first to demonstrate activation of NF-κB binding just 10 minutes into the commonly employed hepatocyte isolation procedure. It is further reported that the anti-oxidant Trolox can prevent the induction of NF-κB during the well established hepatocyte isolation procedure but not during their subsequent culture. However both phases of NF-κB activation are inhibited by *L-NAME* intimating a role for NO production, via nitric oxide synthase. These findings demonstrate that at least 2 different signal transduction pathways are operative during hepatocyte isolation and culture. Thus further studies employing Trolox and *L-NAME* will help delineate how each pathway contributes to the generalised loss of liver function commonly observed *in vitro*. © 1999 Academic Press

Primary cultures of hepatocytes are widely used in biochemical research [1] and form the mainstay of bioartificial liver support systems employed in clinical medicine [2]. In both applications their usefulness is limited by a rapid loss of function [3]. Although research in this laboratory has demonstrated that the spontaneous loss of liver specific cytochromes P450 *in vitro* occurs over 2 distinct time frames the nature of the underlying stimuli are unknown [4-6].

In the current work we demonstrate that NF-κB binding to its consensus sequence in electrophoretic mobility shift assays (EMSA) is the earliest change reported to date occurring just 10 minutes into the conventional hepatocyte isolation procedure. In view of the inhibitory effect of *L-NAME*, this process seems to involve nitric oxide synthase [E.C.1.14.13.39] but how this relates to the loss of hepatocyte phenotype *in vitro* remains to be determined.

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Abbreviations used: *L-NAME,* Nω-Nitro-L-Arginine Methyl Ester; *D-NAME,* Nω-Nitro-D-Arginine Methyl Ester; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; EMSA, Electrophoretic Mobility Shift Assay; HBSS, Hanks balanced salt solution; IH, isolated hepatocytes just prior to culture; NAC, N-acetylcysteine; DTT, Dithiothreitol; PDTC, Pyrrolidine dithiocarbamate; NGA, Nordihydroguaiaretic acid; NOS, Nitric oxide synthase [E.C. 1. 14. 13. 39].

MATERIALS AND METHODS

Chemicals. Oligonucleotide probes containing the consensus sequence for wild type NF-κB, mutant NF-κB, HNF-3, AP1 were synthesised and purchased from Pharmacia, St Albans. Hertfordshire, U.K.

Antibodies to *rel* family proteins were purchased from Santa Cruz Biotechnology, Los Angeles, California, USA.

Trolox was purchased from Aldrich Chemical Co., Gillingham, Dorset, U.K. and N-acetylcysteine (NAC), dithiothreitol (DTT), pyrrolidine dithiocarbamate (PDTC), nordihydroguaiaretic acid (NGA), Nω-nitro-L-arginine methyl ester (*L-NAME*) and Nω-nitro-D-arginine methyl ester (*D-NAME*) were from Sigma, Poole, Dorset, U.K.

Hepatocyte isolation and culture. Hepatocytes were isolated from adult male Sprague-Dawley rats (250-290g) supplied by Charles River Ltd., Margate, Kent, UK. Following anaesthesia resulting from intraperitoneal injection of 60 mg of sodium pentobarbitone/kg body weight (Saggatal; May & Baker, Dagenham, Essex, UK) the liver was cleared of blood by perfusion with Ca^{2+} and Mg^{2+} free Hanks balanced salt solution (HBSS) [Flow Laboratories, Irvine, Scotland, U.K.] gassed with 95% O_2 5% CO_2. The caudate lobe was ligated and removed to provide a sample of intact liver [designated liver lobe in the figures] prior to dissociation into single hepatocytes. Then hepatocytes were isolated by perfusion for 30 min with Ca^{2+} and Mg^{2+} free HBSS gassed with 95% O_2 and 5% CO_2 containing 0.025% (w/v) collagenase H (Boehringer, Lewes, E. Sussex, UK). When samples were taken at timed intervals during the cell isolation procedure this was done by ligating and removing an entire liver lobe. NF-κB binding activity was not observed in any lobe prior to collagenase perfusion and therefore its appearance does not represent inter lobular differences in expression. After 30 min of perfusion the liver was dissociated into a cell suspension by gentle agitation in Ca^{2+} and Mg^{2+} free HBSS containing 2% (w/v) bovine serum albumin (Sigma, Poole, Dorset, UK) followed by filtration through a 125 μm pore size Nylon mesh (Nybolt no. 10.5; John Stanair & Co., Whitefield, Manchester, UK) and centrifugation at 50g for 3 min. The pellet was resuspended in 75-80ml of culture medium comprising serum-free Williams medium E (Flow Laboratories, Irvine, Scotland, UK) containing 50 μg gentamicin/ml, 10^{-6} M insulin, 10^{-4} M hydrocortisone-21-sodium succinate (all from Sigma). A further three washing and

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centrifugation steps were performed before the cells were resuspended in 35 ml of serum free Williams medium E. An aliquot was diluted 1:10 in phosphate buffered saline pH 7.4 (Flow Labs) containing 0.1% (w/v) Trypan Blue (Sigma) and the cell number and viability enumerated using a haemocytometer. The viability of hepatocytes, as assessed by Trypan Blue exclusion, was routinely greater than 85%. Cells (2×10^7) were suspended in 20 ml of serum free culture medium containing 1 μg bovine plasma fibronectin/ml (Sigma, Poole, Dorset, UK) and added to 150 mm-diameter Lux Scientific plastic Petri dishes purchased from Flow Laboratories. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Preparation of cell extracts for EMSA. Whole cell extracts were prepared from liver lobes, isolated and cultured hepatocytes essentially as described by Shreck and Baeuerle [7] for lymphocytes.

Intact liver lobes (1g. samples) were homogenised in 4 ml of homogenisation buffer [20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 2 mM dithithreitol (DTT), 10 μg/ml leupeptin, 10 μg/ml aprotinin which contained 0.5% (v/v) of a saturated phenylmethylsulfonyl fluoride (PMSF) solution in ethanol] by 5 up and down strokes of a Potter type motor driven (1500 rpm) homogeniser. The resulting homogenate was diluted 1:2 in 2× Lysis Buffer [40 mM HEPES-KOH, pH 7.5, 700 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.2 mM EGTA, 10 mM DTT, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 1% (v/v) of a saturated PMSF solution in ethanol, 40% (v/v) glycerol, and 2% (v/v) Nonidet P-40 (NP-40)].

Freshly isolated (IH) or cultured hepatocytes (2×10^7) were collected either directly from the cell suspension prior to plating or by scraping attached hepatocytes into the culture medium and immediate centrifugation at 2,300g for 3 mins. The cell pellet was resuspended in 1ml of ice-cold PBS and cells sedimented again by centrifugation at 2,300g for 3 mins. The cell pellet was resuspended in 10 μl 1× Lysis Buffer per 10^5 cells.

After 15 min on ice, lysates both from liver lobes and cells were centrifuged at 4°C at 15,800g for 20 mins. The supernatant was diluted with 1 volume of dilution buffer [20 mM HEPES-KOH, pH 7.9, 0.2 mM EDTA and 20% (v/v) glycerol] in order to reduce the salt concentration. The extracts were aliquoted, quick-frozen in liquid nitrogen and stored at −70°C until use. The average protein concentration of extracts was between 7-13 μg/μl, as determined by a Bradford microassay procedure (Bio-Rad, Richmond, CA, USA). Equal amounts of protein (20 μg) were used in DNA-binding reactions for EMSAs.

EMSA assays. To monitor the presence of NF-κB binding activity in the cell extracts the following probes and competitors used were: NF-κB (wild type sequence, underlined, derived from human immunoglobulin gene [8]) 5′-ACAGAGGGGACTTTCCG-3′ and its complementary strand 5′-CCTCTCGGAAAGTCCCC-3′. NF-κB (mutant type, base substitution underlined) 5′-ACAGAGGCGACTTTCCG-3′ and 5′-CCTCTCGGAAAGTCGCC-3′, AP1 (from the mouse alpha-1-antitrypsin gene [9]) 5′-AGAGGGCCATGTGACTCATTAC-3′ and complementary 5′-CTGGTGTAATGAGTCACATGGC-3′. HNF-3 (from the mouse transthyretin gene [10]) 5′-TGACTAAGTGAATAATCAGAATCAG-3′ and complementary 5′-ACCTGCTGATTCTGATTATTGACTT-3′. The probes were terminally labelled with [^{32}P] dNTPs and annealed using the Klenow fragment of DNA polymerase I (Promega, Southampton, U.K.) and purified using NucTrapR columns (Stratagene, Cambridge, U.K.). For the binding assay, 20 μg of protein extract and 2 μg of poly (dI-dC) (Pharmacia St Albans, Herts, U.K.) were incubated in 7.5 μl of 2× binding buffer [10 mM HEPES pH 7.9, 10% glycerol, 3 mM $MgCl_2$, 60 mM KCl, 1 mM DTT] for 10 min at room temperature. Then, the binding reaction was started by adding 10,000 cpm of the ^{32}P-labelled dsDNA probe and incubated for 20 min on ice. In the supershift assays employing antibodies (all from Santa Cruz Biotechnology, CA, USA) the antibody was added at 4°C 1 hour before the probe. Protein-DNA complexes were resolved using a non-denaturing polyacrylamide gel consisting of 5% (w/v) polyacrylamide and 0.25% (w/v) bis-acrylamide run in 0.045 M Tris-borate buffer pH 8.0 containing 1 mM EDTA for 2 h at 150 mV. Gels were transferred to Whatman 3M paper, dried under vacuum at 80°C, and exposed overnight to X-ray film (Kodak Biomax MS, Eastman Kodak Company) at −70°C with an intensifying screen.

RESULTS AND DISCUSSION

Characteristics of NF-κB Binding Activity

The results presented in Fig. 1a demonstrate that the binding of a protein in hepatocyte extracts to a 17 mer probe containing the NF-κB consensus becomes apparent, in electrophoretic mobility shift assays (EMSA), just 10 minutes into the commonly employed liver cell isolation procedure. This effect persists, when hepatocytes are cultured, for at least the next 2 hours but is absent after 24 hours of culture (Fig. 1a). That this binding activity represents NF-κB is supported by the results presented in Fig. 1b which demonstrate that 100 fold molar excess of unlabelled NF-κB probe completely competes out the binding properties of hepatocyte extracts to the ^{32}P labelled NF-κB probe. In contrast a 100 fold molar excess of unrelated oligonucleotides encoding the consensus sequences for AP1 or HNF-3 are ineffective competitors. Furthermore binding is not competed when an unlabelled mutant NF-κB probe differing in just one nucleotide (see Materials and Methods section for sequence) is employed at 100 fold excess (Fig. 1b). Conversely use of the radiolabelled mutant probe in EMSA with hepatocyte extracts does not result in a species being retarded from the "free" probe (data not shown for brevity). Finally the results presented in Fig. 1c show that the binding property of hepatocyte extracts can be "supershifted" by an antibody to the p65 subunit of NF-κB. Antibodies to other *rel* family members are ineffective in "supershift" experiments suggesting that the binding species comprises p65 homodimers (Fig. 1c).

Effect of Trolox on NF-κB Binding Activity

The results presented in Fig. 2 demonstrate that the incorporation of 1mM Trolox into the hepatocyte isolation medium completely prevents NF-κB binding activity appearing in hepatocyte extracts prepared at any time during the 30 minute liver cell isolation procedure. Similarly washing the hepatocyte preparation prior to culture in medium containing 1 mM Trolox, which takes another 30 minutes, also prevents the induction of NF-κB binding activity in hepatocyte extracts (sample IH in Fig. 2). However 1 hour after culturing the cells in medium containing 1 mM Trolox NF-κB binding activity appears (sample 1 h in Fig. 2). Internal controls to the experimental protocol indicate that the binding species is again NF-κB because binding is competed by 100 fold molar excess of unlabelled NF-κB probe but not by a 100 fold excess of the unrelated AP1 probe (Fig. 2).

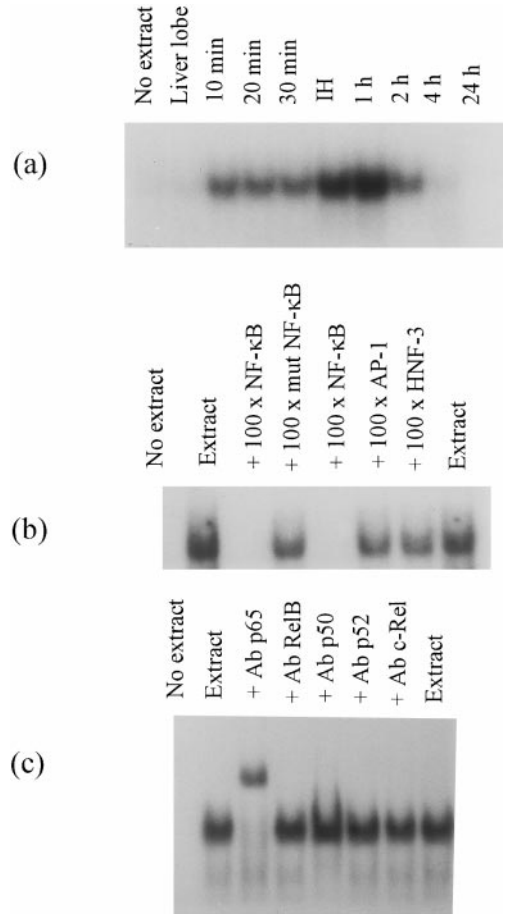

FIG. 1. Characteristics of NF-κB binding activity of hepatocyte extracts. In panel (a) a liver lobe was removed prior to perfusion with collagenase and then after 10, 20 & 30 mins. IH = freshly isolated hepatocytes. 1 h-24 h = time of culture in hours. In panels b & c EMSA used extracts prepared 20 mins into hepatocyte isolation treated with (b) 100 fold molar excess of different probes or (c) antibodies to rel family members. Results representative of 3 individual liver perfusions.

Effect of Other Anti-Oxidants

Pahl and Baeuerle [11] have described a biochemical pathway leading to NF-κB activation by oxidative stress. In their scheme the ability of reactive oxygen intermediates to activate NF-κB can be prevented by N-acetyl cysteine (NAC), dithiothreitol (DTT), pyrrolidine dithiocarbamate (PDTC) and nordihydroguaiaretic acid (NDGA). Accordingly hepatocytes were isolated in medium containing 1 mM NAC, 1 mM DTT, 200 μM PDTC and 100 μM NDGA which was the limit of solubility of the latter two compounds in HBSS. In all cases hepatocyte preparations of good (83-91%) viability were produced but only isolation with 1mM DTT prevented the induction of NF-κB binding activity. When hepatocytes were cultured for 2 hours with the individual anti-oxidant at the same concentrations employed during their isolation, again only 1 mM DTT abrogated NF-κB induction (data not shown for brevity).

Effect of L-NAME

In an attempt to determine if NF-κB induction had an innate enzymatic stimulus we isolated hepatocytes in media containing 500 μM *L-NAME* [as an inhibitor of nitric oxide synthase (NOS)] and found it to be effective as was the lower concentration of 250 μM. Culture of hepatocytes with 250 μM *L-NAME* also blocked the induction of NF-κB binding activity. In contrast the D-enantiomer, which is known not to inhibit NOS [12], was unable to prevent the rise in NF-κB binding that occurs during hepatocyte isolation or culture (Fig. 3). These findings suggest that NO production via NOS is the source of NF-κB activation during both hepatocyte isolation and culture.

Properties of the Second Wave of NF-κB Binding Activity

The results presented in Fig. 2 demonstrate that Trolox is only able to block NF-κB binding during the hepatocyte isolation procedure but not during their subsequent culture for 1 hour. However *L-NAME* was found to be effective at preventing the activation of NF-κB occuring during both hepatocyte isolation and culture (Fig. 3) indicating that in both situations NF-κB activation proceeds via NOS. Since Trolox is known to scavenge peroxynitrite [13], formed through the interaction of superoxide and NO, the different sensitivities of NF-κB induction to Trolox during hepa-

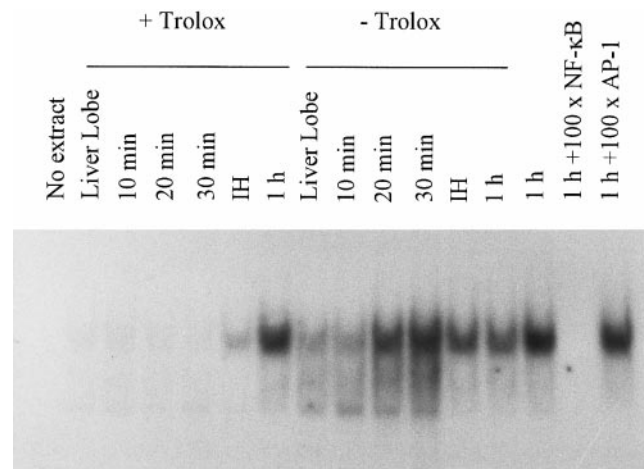

FIG. 2. Trolox prevents NF-κB activation during hepatocyte isolation. Hepatocyte isolation was performed with (+) or without (−) 1mM Trolox in Ca^{2+} and Mg^{2+} free HBSS gassed with 95% O_2 and 5% CO_2. The caudate lobe was removed before addition of 0.025% (w/v) collagenase to provide a t_0 (liver lobe) value. Then whilst perfusing with collagenase individual liver lobes were removed following ligation at 10, 20 and 30 mins. IH = hepatocytes immediately prior to culture. 1 h = hepatocytes after 1 hour of culture in serum free Williams E containing 1 μg bovine plasma fibronectin/ml. The results presented are from single experiments with and without Trolox and are representative of 3 individual liver perfusions with and without Trolox.

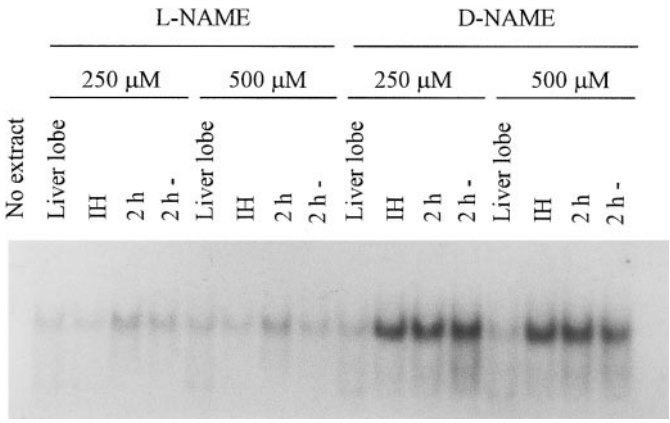

FIG. 3. L-NAME, but not D-NAME, blocks NF-kB activation during the isolation and culture of rat hepatocytes. Hepatocyte isolation was performed with 250 μM and 500 μM L- or D-NAME in Ca^{2+} Mg^{2+} free HBSS gassed with 95% O_2 and 5% CO_2. The caudate lobe was removed before addition of 0.025% (w/v) collagenase to provide a t_0 (liver lobe) value. IH = hepatocytes immediately prior to culture. 2 h samples = hepatocytes from 2 separate liver cell preparations cultured with L- or D-NAME for 2 hours.

tocyte isolation and culture suggests the involvement of peroxynitrite during the cell isolation proceedure and just NO during culture.

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