TPA and cycloheximide modulate the activation of NF-κB and the induction and stability of nitric oxide synthase transcript in primary neonatal rat hepatocytes

Marta Menegazzi[a], Chiara Guerriero[b], Alessandra Carcereri de Prati[a], Caterina Cardinale[b], Hisanori Suzuki[a], Ubaldo Armato[b,*]

[a]*Institute of Biochemistry, Medical School, University of Verona, Strada Le Grazie 8, I-37134 Verona, Italy*
[b]*Institute of Anatomy and Histology, Medical School, University of Verona, Strada Le Grazie 8, I-37134 Verona, Italy*

Received 10 November 1995

Abstract 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) elicited a transient increase in the transcription of the inducible nitric oxide synthase (iNOS) gene coupled with a shortening of the half-life of its mRNA in primary neonatal rat hepatocytes. These effects of TPA were preceded by a surge in nuclear translocation of the transcription factor NF-κB, and followed by a mounting accumulation of NO_2^- in the growth medium. Even cycloheximide (CHX) added by itself elicited an early, sustained activation of NF-κB followed by an intense induction of iNOS gene expression, irrespective of what degree of protein synthesis inhibition was brought about by the several concentrations tested. When given together, TPA and CHX exerted additive effects on hepatocellular iNOS mRNA levels. These results suggest the likelihood of an ordered sequence of events by which an activated NF-κB mediates the induction of iNOS gene expression in TPA- and/or CHX-treated primary hepatocytes.

Key words: Primary liver culture; Gene expression; Nitric oxide synthase; NF-κB; 12-*O*-Tetradecanoylphorbol 13-acetate; Cycloheximide

1. Introduction

Several lines of evidence attest the involvement of the nitric oxide (NO) pathway in the functional regulation of any organ and tissue in the body [1–3]. This gaseous compound performs its complex tasks by acting as an intra- or extracellular messenger molecule [2,4]. NO is generated from L-arginine by the activity of distinct enzymes, i.e. two constitutive and one inducible synthases (cNOS and iNOS, respectively), which may not be all expressed in the same cells [1]. Moreover, each type of tissue or cell appears to be endowed with a distinct assortment of mechanisms governing the induction of iNOS gene expression [1,3]. As to the liver, some cNOS activity is normally detectable in Kupffer cells, whereas no cNOS is ever encoded in hepatocytes [5]. On the other hand, both main types of hepatic cells and Ito cells too are prompted to express an intense iNOS activity once exposed to effective stimuli, e.g. bacterial lipopolysaccharide (LPS) along with mixtures of cytokines such as tumor necrosis factor-α, interleukin-1, and interferon-γ (IFN-γ) [6–8]. A chronic inflammatory process striking the liver also effectively induces iNOS activity [9]. The import of NO biosynthesis by Kupffer cells and by hepatocytes in key pathophysiological processes, such as inflammation and defence against the invasion by infectious micro-organisms or malignant tumour cells, is only beginning to be clarified [5,10].

Various studies have reported the presence of two binding sites for the transcription factor NF-κB in the sequence of the promoting region of iNOS gene, thereby implicating such a factor in the modulation of its transcription in cell types unrelated to the liver [11–14]. Notably, NF-κB is constitutively existent as an inactive cytoplasmic heterotrimeric complex that can be activated by proper stimuli via a post-translational phosphorylation mechanism involving several kinases – but no new protein synthesis – leading to the dissociation of NF-κB from cytosolic IκB proteins; released NF-κB is then able to translocate into the nucleus, where it binds to specific DNA sequences [13–15]. The role(s) played by NF-κB in the modulation of the expression of iNOS gene by mammalian hepatocytes still remain(s) to be fully clarified.

Primary cultures of adult hepatocytes were used as a model system to investigate some facets of the process of iNOS induction by mixtures of LPS with cytokines [9,16,17]. Conversely, other types of reportedly effective inducers (e.g. phorbol esters) have hitherto attracted much less attention [18]. Moreover, the likelihood that iNOS inducing agents and/or the susceptibility to them of either hepatocytes or Kupffer cells or of both may vary along with the several stages of life has hitherto been, as far as we know, neglected. Therefore, we endeavoured to throw further light onto the mechanisms modulating the induction of iNOS activity elicited by the well-known tumor promoting agent TPA by using as an experimental model neonatal rat hepatocytes set into pure primary cultures. In previous papers, we showed that TPA enhances the proliferation of the primary neonatal hepatocytes by activating a rather complex panel of operative mechanisms [19,20]. In this communication, we report that TPA and the protein synthesis inhibitor CHX can both function, either singly or in an additive fashion, as fully effective super-inducers of iNOS gene expression in neonatal rat hepatocytes kept in synthetic growth medium. Additionally, an increased translocation of NF-κB into the nuclei antecedent

*Corresponding author. Tissue Culture Laboratory, Institute of Anatomy and Histology, Strada Le Grazie 8, I-37134 Verona, Italy. Fax: (39) (45) 8098 170.

Abbreviations: Act. D, actinomycin D: CHX, cycloheximide: cNOS, constitutive NO synthase; EGF, epidermal growth factor; EMSA, electrophoretic mobility shift assay; FBS, foetal bovine serum; IFN-γ, interferon-γ; iNOS, inducible NO synthase; LPS, (bacterial) lipopolysaccharide; MEM, minimum essential medium; NO, nitric oxide; PDTC, pyrrolidine dithiocarbamate; TGF-β, transforming growth factor-β; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

SSDI 0014-5793(95)01527-2

the enhancement over baseline levels of the transcription of iNOS mRNA on the part of TPA and/or CHX has been observed for the first time in the same hepatocytes, suggesting that these two processes may be tightly connected.

2. Materials and methods

2.1. Chemicals

All the chemical agents used were from Sigma Chemical Co. (St. Louis, MO), unless otherwise specified. All solutions and growth media were made up with ultrapure water produced by a combined Milli-Ro and Milli-Q UF system (Millipore S.p.A., Milan).

2.2. Neonatal rat hepatocyte and HeLa cell cultures

Livers were removed from 2- to 4-day-old Wistar rats (S. Morini, S.a.s., San Polo d'Enza, RE), pooled, and set into primary cultures as previously detailed [21]. Briefly, neonatal liver cells were isolated both mechanically and enzymatically, and hepatocytes were next separated from blood and stromal cells, dead cells, cell debris, collagen fibers, and large cell clumps in a Percoll (Pharmacia S.p.A., Milan) gradient [21]. The purified hepatocytes (96–98% of the cell total population) were resuspended in synthetic $HiWo_5Ba_{2000}$ medium, which contained 1.0 $\times$ 10^{-3} M Ca^{2+} and 7.5 mg/l ornithine instead of arginine [21]. Just before use, 50 IU/ml streptomycin (Squibb & Sons S.p.A., Rome), 5.35 μg/ml imipenem monohydrate (Sigma Tau S.p.A., Rome), 5.35 μg/ml sodium cilastatin (Sigma Tau), and 250 ng/ml fungizone (Mascia-Brunelli S.p.A., Milan) were added to this medium. Once their numbers had been assessed, the hepatocytes were diluted to a final concentration of 1.0 $\times$ 10^6/ml, and planted in 6-well cluster plates (Costar Italia, S.r.l., Milan) at 0,5 x 10^6 cell/well. At the bottom of each well was a non-toxic, ultrathin (37.5 μm), porous polyethylene disc (Visqueen Ltd., London, UK), the upper cell-receiving surface of which had been coated with a film of type I collagen. Before planting the hepatocytes, the collagen-coated discs were covered with a culture medium consisting of 90% v/v $HiWo_5Ba_{2000}$ medium and 10% v/v inactivated, dialysed fetal bovine serum (FBS, ICN-Flow S.p.A., Milan), which contained 10 ng/ml epidermal growth factor (EGF), 100 pM glucagon, 5.0 μg/ml insulin, and 0.4 μg/ml hydrocortisone, as well as 1.12 g/l $NaHCO_3$. The cultures were incubated at 37°C in humidified 95% air/5% CO_2 mixture. At 24 hours, the porous discs onto which the hepatocytes had been planted were removed from the wells and floated cell-side down on the surface of a fresh medium consisting of 5% v/v FBS and 95% v/v Eagle's Minimum Essential Medium (MEM) with $NaHCO_3$ but *without* EGF, glucagon, hydrocortisone, and insulin. At 48 hours, this medium was discarded and replaced with serum-free and hormones-free Eagle's MEM medium with $NaHCO_3$.

HeLa cells were cultured under standard conditions in Dulbecco's modified MEM medium (Gibco-BRL S.p.A., Milan) fortified with 10% (v/v) FBS, antibiotics, and glutamine.

2.3. Experimental procedures

Experiments using primary neonatal rat hepatocyte cultures were started on the fourth day of staying in vitro. Control specimens were exposed to fresh, serum-free, EGF- and hormones-free MEM medium. The experimental cultures were divided into several groups and received a change of the same medium as used for the controls except for containing TPA (10^{-9} M). In another set of experiments, several doses of either CHX (from 0.25 to 25 μg/ml) or pyrrolidine dithiocarbamate (PDTC; from 10^{-8} to 10^{-3} M) – a reputedly specific inhibitor of NF-κB [13] – were added to the fresh synthetic medium 15 min and 1 hour, respectively, before administering TPA to the primary hepatocytes. As shown in a pilot experiment, CHX used singly at 25 μg/ml totally inhibited the macromolecular incorporation of [^{3}H]leucine by the primary hepatocytes, whereas CHX at 0.25 μg/ml was unable to change the rate of ongoing protein synthesis in the same cells. In further experiments, actinomycin D (Act. D, 5.0 μg/ml) was administered to both untreated (control) and TPA-treated (10^{-9} M for 3 hours) liver cultures. This dose of Act-D completely hindered the macromolecular incorporation of [^{3}H]uridine by the cultured hepatocytes in a pilot experiment.

Primary neonatal hepatocyte cultures were sampled at predetermined times after the onset of the various treatments, and the corresponding total cellular RNA's and nuclear extracts were prepared as detailed below. NO_2^- concentrations were also determined in the conditioned growth media pertaining to some of the above experiments.

Confluent HeLa cell cultures were divided into three groups: the first received only a change of fresh medium; the second was exposed to 5.0 $\times$ 10^{-8} M TPA for 1.0 hour; and, the third was preincubated for 1 hour with PDTC (from 1.0 $\times$ 10^{-6} to 2.0 $\times$ 10^{-4} M) before adding 5.0 $\times$ 10^{-8} M TPA. Nuclear extracts were prepared from the specimens thus collected (see below).

2.4. Northern blot analysis

Total RNA was extracted by the guanidine thiocyanate/cesium chloride method [22]. Samples of 20 μg of total RNA were separated by electrophoresis on 1.0% agarose gel in MOPS buffer, blotted into a Hybond N membrane (Amersham, Milan), and hybridised with the cDNA either for rat iNOS [23] or for *v-fos* previously labelled by means of a Megaprime DNA labelling system (1.0–2.0 $\times$ 10^9 cpm/μg; Amersham). The amounts of specific mRNA were measured in a Phosphor

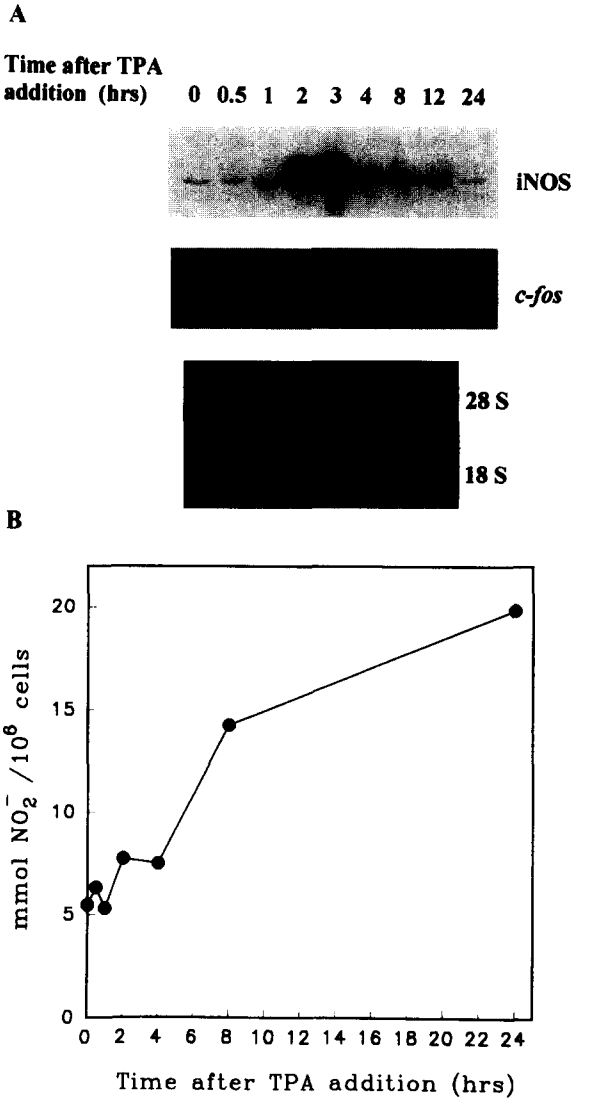

Fig. 1. (A) The induction of iNOS and of *c-fos* gene expression by TPA in primary neonatal rat hepatocytes. Details on the culture procedure, experimental treatment, and Northern analysis are given in section 2. The bottom panel shows the ethidium bromide staining of the same gel shown both in the intermediate and top panels prior to undergoing the blotting procedure. 28 S and 18 S mark the levels of the corresponding ribosomal RNA species. (B) The time-related changes in the amount of NO_2^- assayable in the synthetic MEM medium per million of primary neonatal rat hepatocytes after the onset of the exposure to TPA (10^{-9} M). The points are the mean values from two distinct experiments.

Imager SF (Molecular Dynamics, Milan). In those experiments in which iNOS mRNA half-lives were assessed, control and 3-hour TPA-pre-pretreated rat hepatocytes were incubated with Act. D (5.0 μg/ml) for up to 4 hours, and the declining steady-state levels of the specific mRNA were monitored by Northern analysis in the corresponding samples.

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to Schreiber et al. [24] in the presence of 10 μg/ml leupeptin, 5.0 μg/ml antipain, 5.0 μg/ml pepstatin, and 1.0 mM PMSF. The protein concentration of the nuclear extracts was determined according to Bradford [25]. A sample (8.0 μg) of each nuclear extract was incubated at room temperature for 30 min with either of two ^{32}P-labelled double stranded oligonucleotides (0.2 ng, corresponding to about 2.0–5.0 × 10^4 cpm), of which one contained a consensus NF-κB DNA binding site (5′-GATCCAGAGGGGACTTTCCGAGTAC-3′; a gift from Dr. M. Merola) and the other an AP-1 DNA binding site (5′- CTAGTGATGAGTCAGCCGGATC-3′; a gift from Dr. G. Rigaud), in 15 μl of a reaction mixture made up by 20 mM HEPES pH 7.9, 50 mM KCl, 0.5 mM DTT, 0.1 mM EDTA, 2.0 μg poly(dI-dC) (Pharmacia S.p.A., Milan), 1.0 μg salmon sperm DNA, and 10% glycerol. Products were fractionated on a non-denaturing 5% polyacrylamide gel. In competition assays, a 100-fold amount of 'cold' oligonucleotide competitor was added 15 min ahead of administering the respective labeled probe. The corresponding intensities of the retarded bands were measured in a Phosphor Imager SF (Molecular Dynamics, Milan).

2.6. Determination of NO_2^- in the growth media

NO_2^- concentrations were assayed in phenol red-free hepatocyte-conditioned growth media at various time points after the addition of TPA (10^{-9} M). After reacting with Griess's reagent [26], a 400 μl aliquot of each sample was mixed with an equal volume of 1.0% sulphanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine-dihydrochloride; following a 10 min incubation at room temperature, the optical absorbance values of the samples were read at 540 nm in a DU 7 spectrophotometer (Beckman, Milan), and next correlated with the actual hepatocytes' numbers in the corresponding cultures on floating polyethylene discs.

3. Results

3.1. Super-induction of iNOS mRNA by TPA in primary neonatal rat hepatocytes

Neonatal rat hepatocytes set into pure primary cultures kept in synthetic MEM medium were consistently found, after 4 days of staying in vitro, to spontaneously express discrete basal levels of iNOS mRNA (Fig. 1A, top panel). The addition of TPA (10^{-9} M) to this same medium could elicit a striking increase (i.e. mean: 6-fold, and range: from 4- to 20-fold in 8 distinct experiments) in the steady-state levels of the hepatocytic iNOS transcript, which peaked between 2 and 3 hours, slowly dwindled thereafter, and returned to the spontaneous baseline values by about 24 hours (Fig. 1A, top panel). When the same blots used to detect iNOS mRNA signals were re-hybridized with a *v-fos* radioactive cDNA probe, it became manifest that the untreated primary hepatocytes did not express the *c-fos* transcript at all, whereas the TPA-exposed ones exhibited the general kinetics of *c-fos* mRNA induction, that is a discernible *c-fos* signal peaking at 30 min and totally disappearing after the first hour (Fig. 1A, intermediate panel).

3.2. Half-lives of basal and TPA-stimulated hepatocellular iNOS transcripts

As the TPA-elicited surge of iNOS mRNA we observed might have been brought about by a lengthening of the half-life of the spontaneously expressed transcript rather than by a true super-induction of this gene, the half-lives of iNOS mRNA's had to be assessed under both untreated and phorbol ester-treated conditions. Thus, the spontaneously expressed iNOS transcript was found to dwindle rather slowly and to have a calculated half-life of 7.2 hours in the control hepatocytes, whereas its levels declined much more rapidly in TPA-pre-incubated hepatocytes with a half-life of 1.9 hours only (Fig. 2). Hence, TPA simultaneously exerted two distinct actions, since it both super-induced the expression of the iNOS gene and remarkably decreased the half-life of its transcript.

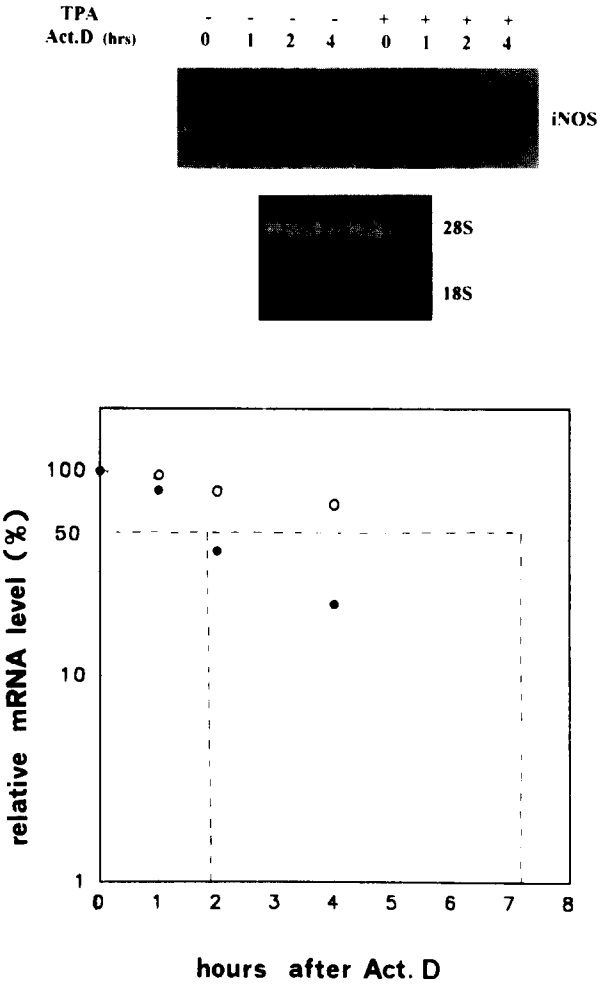

Fig. 2. Top panel. Northern blot analysis of iNOS transcript after the addition of Act. D (5.0 μg/ml) for up to 4 hours to the medium of either untreated or 3-hour TPA-pre-treated pure primary cultures of neonatal rat hepatocytes. Intermediate panel. The ethidium bromide staining of the corresponding gel prior to its blotting is shown. 28 S and 18 S are as in Fig. 1. Bottom panel. Semilogarithmic plot of the decay rates of iNOS transcripts in untreated (○) and in TPA-treated (●) primary hepatocytes after the addition of Act. D (5.0 μg/ml). The corresponding half-lives are 7.2 hours for the controls and 1.9 hours for the 3-hour TPA-pre-treated specimens, respectively. The points are the means from three different experiments.

3.3. Levels of NO_2^- in primary hepatocytes' growth media

The basal production of NO by the untreated (control) hepatocytes and its subsequent diffusion into the extracellular environment were mirrored by the steady presence of discrete levels of NO_2^- in the conditioned synthetic media to which the parenchymal liver cells had been exposed (Fig. 1B). The addition

of TPA (10^{-9} M) nearly did not change, with respect to parallel controls, the media's NO_2^- concentrations in the course of the first 4 hours; subsequently, the NO_2^- levels increased swiftly between the 4th and the 8th hour and rather more gradually up to the 24th hour (Fig. 1B).

3.4. Super-induction of iNOS gene expression by CHX in primary neonatal rat hepatocytes

To throw light onto the operative mechanism(s) underlying the TPA-elicited enhancement of iNOS mRNA transcription, we endeavoured to find out whether this action of the phorbol ester did depend upon the de novo synthesis of some specific protein(s). Thus, a 15 min pre-treatment of the primary hepatocytes with a concentration of CHX (i.e. 25 μg/ml) fully inhibiting their protein synthesis could not impede the subsequent super-induction of iNOS transcript evoked by TPA (10^{-9} M) (Fig. 3A). Unexpectedly, the same CHX dose given by itself was found to be able to induce iNOS gene expression and, moreover, to exert, when added along with TPA, an almost additive enhancing effect on the steady-state levels of iNOS transcript (Fig. 3A). Thus, by averaging the results of three distinct experiments, TPA (10^{-9} M) by itself elicited a 6-fold increase (over controls' baseline) in iNOS mRNA steady-state levels; CHX used alone (25 μg/ml) effected an 11-fold increase of this same transcript, and the corresponding TPA plus CHX mixture elicited a 15-fold increase of this mRNA, that is a nearly additive outcome. Moreover, two lower concentrations (i.e., 2.5 and 0.25 μg/ml) of CHX alone were also tested and found to super-induce iNOS mRNA transcription as effectively as did the 10-or 100-fold higher dose of CHX (Fig. 3B), even though when given at 0.25 μg/ml this agent did not change the rate of ongoing protein synthesis in hepatocytes (data not plotted).

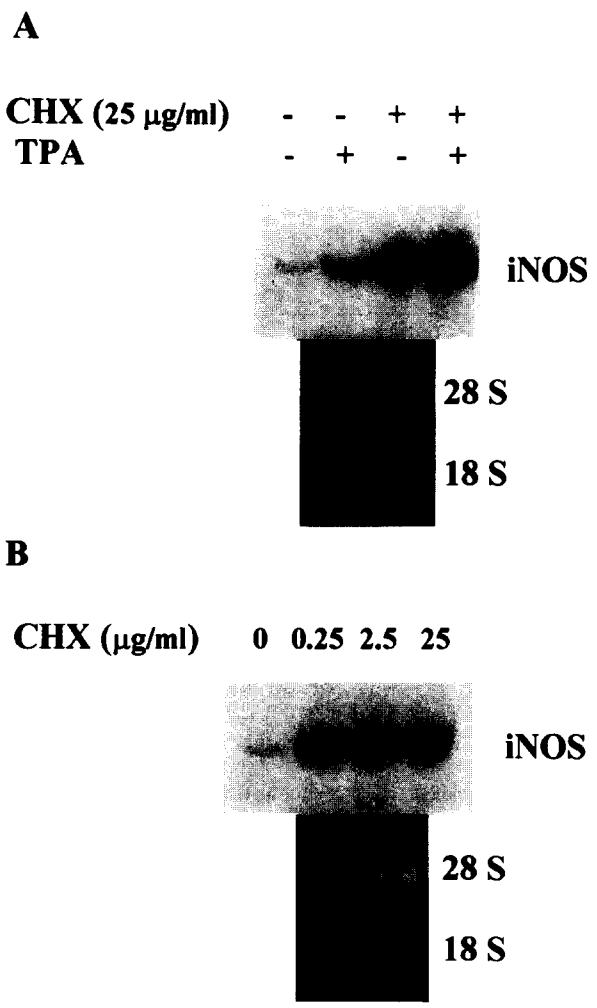

Fig. 3. (A) Both the super-induction of iNOS gene expression by either TPA (10^{-9} M) or CHX, each used by itself, and the additive induction of the same transcript brought about by the association of CHX with TPA in the primary neonatal rat hepatocytes are shown here as the results of one representative experiment. (B) The nearly equivalent effectiveness of three different doses of CHX on the super-induction of iNOS gene expression in primary hepatocytes. In both panels A and B, the ethidium bromide stainings of the corresponding gels are also shown. 28 S and 18 S are as in Fig. 1.

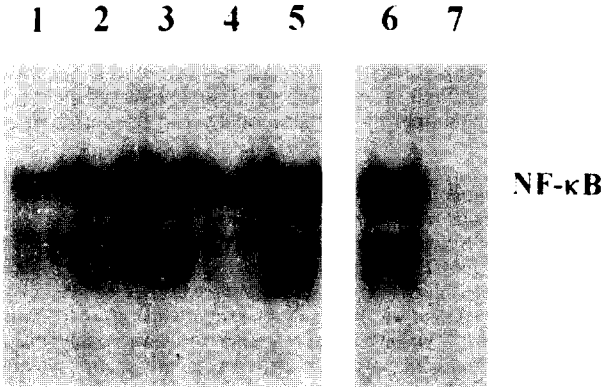

Fig. 4. EMSA analysis of nuclear extracts of primary neonatal rat hepatocytes showing the activation of NF-κB occurring spontaneously or brought about by an exposure to either TPA (10^{-9} M) or CHX (25.0 μg/ml). Lane 1, untreated parallel controls; lanes 2–4, specimens treated with TPA for 0.5, 1.0, and 2.0 hours, respectively; lane 5, specimen exposed to CHX for 2.0 hours; lane 6, a 100-fold concentration of non-specific unlabelled oligonucleotide (e.g., AP-1) was added to nuclear extracts treated as those in lane 3, 15 min prior to the administration of the specific labelled probe; lane 7, a 100-fold concentration of the specific unlabelled oligonucleotide was added to the nuclear extracts treated as those in lane 3 15 min prior to the administration of the specific labelled probe.

3.5. Nuclear transcription factors and iNOS gene expression

A basal level of activated, i.e. DNA-bound NF-κB was detectable by EMSA in the nuclear extracts of untreated (control) primary neonatal rat hepatocytes (Fig. 4, lane 1) that were also simultaneously expressing basal steady-state levels of iNOS mRNA (Fig. 1A, top panel). The exposure to TPA (10^{-9} M) further increased the level of activated NF-κB in the nuclear extracts of the primary hepatocytes, an effect peaking at 1 hour and completely vanishing by 2 hours (Fig. 4, lanes 2–4). Besides, even CHX (25 μg/ml) used by itself was found to elicit an extra increase in the translocation of NF-κB into the nucleus: yet, at variance with TPA's action, this effect of CHX was still close to its peak at 2 hours (Fig. 4, lane 5).

The anti-oxidant agent PDTC has been widely used as a specific inhibitor of NF-κB activation in various kinds of non-hepatic cells [13,27–29]. Surprisingly, none of the several doses (from 10^{-8} to 10^{-3} M) of PDTC we tested by adding them to the synthetic MEM medium 1 hour earlier than TPA (10^{-9} M) could suppress either the prior activation of NF-κB (Fig. 5, left) or the later induction of iNOS gene expression in the primary

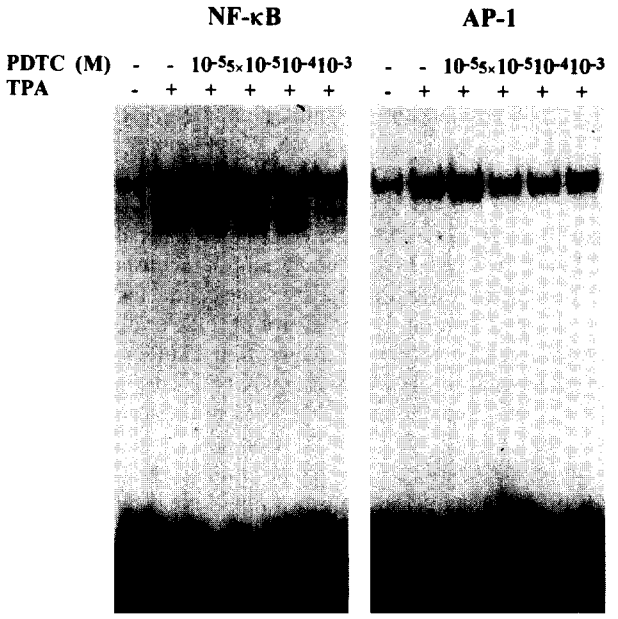

Fig. 5. The inability of several concentrations of the anti-oxidant agent PDTC, added to the growth media of primary hepatocytes 1 hour prior to the onset of the exposure to TPA, to hinder the activation of NF-κB brought about by the phorbol ester (left) and, conversely, the ability of a single dose (i.e. 10^{-5} M) of PDTC to super-activate AP-1 in the same cells (right). It should be noted that even other doses of PDTC besides those shown here, that is from 10^{-6} M to 10^{-8} M, were also similarly tested and found to be unable to change the TPA-elicited activation of NF-κB in neonatal hepatocytes (data not shown).

rat hepatocytes (data not shown). However, one of the PDTC concentrations tested (i.e. 1.0×10^{-5} M) was found to further enhance the activation of AP-1 transcription factor evoked by TPA in the same hepatocytes (Fig. 5, right). At variance with its actions in the rat hepatocytes, PDTC given at doses of 1.0×10^{-4} M or 2.0×10^{-4} M could fully bar the activation of NF-κB otherwise elicited by TPA alone (5.0×10^{-8} M) in HeLa cells (Fig. 6, left). Moreover, when administered at either 5.0×10^{-5} M or 1.0×10^{-4} M, PDTC was also found to further enhance the TPA-evoked activation of AP-1 factor in the same HeLa cells (Fig. 6, right).

4. Discussion

The present results show that both the archetypal tumor promoting phorbol ester TPA and the notorious protein synthesis inhibitor CHX can efficiently boost both the nuclear translocation of NF-κB and the expression of iNOS gene in primary neonatal rat hepatocytes.

To begin with, our findings confirm the previous observation that untreated primary hepatocytes spontaneously express low, yet discrete basal levels of iNOS transcript [30]. As ultrapure water was used to clean any piece of equipment and to make up any growth medium or solution coming into contact with these cells, it is conceivable that the spontaneous iNOS gene expression observed was not brought about by the exposure to any contaminating bacterial endotoxin(s). Because the neonatal rat liver tissue was previously found by us to transcribe no iNOS mRNA in vivo [30], we suspect that the artificial condition(s) inherent to the in vitro environment operate as weak inducer(s) of the hepatocytic iNOS gene. Furthermore, the unvarying presence of a fraction of actively cycling neonatal hepatocytes in the primary cultures [19,20] is unlikely to be in any way related to the basal expression of iNOS transcript, as a growth fraction of similar size is also constantly detectable in the neonatal rat liver tissue in vivo, which normally does not have any iNOS transcript [30]. Moreover, we wish to s tress that the primary neonatal hepatocytes were experimentally used in the complete absence of the serum. Hence, an intracellular repressor induced by a serum factor may conceivably present the basal expression of hepatocytic iNOS gene in vivo. Another possibility not to be overlooked is that in vivo non-parenchymal liver cells produce a paracrine factor suppressing the iNOS gene expression in hepatocytes.

The present results also show that TPA by itself significantly, even though transiently, amplified the basal expression of iNOS gene in the primary neonatal hepatocytes. Notably, TPA could act as a complete iNOS mRNA-inducing factor, not needing any co-operative agent, in these purified hepatocytic cultures, in which the fraction of non-parenchymal cells did not exceed a 2–4% of the total cellular population. Therefore, the superinduction of iNOS gene expression and the subsequent increase in the synthesis and extracellular liberation of NO elicited by the phorbol ester were all neonatal hepatocyte-specific responses. The sharp increase in the rate of accumulation of NO_2^- in the serumless growth media we observed to occur between the 4th and the 8th hour of treatment with TPA is consistent with a heightened level of enzymatic activity due to an expanded population of iNOS molecules encoded under an increased availability of their specific transcript. On the whole, these findings are in keeping with alike inducing effects on iNOS activity elicited by TPA in primary cultures of adult rat hepatocytes, as reported by Hortelano et al. [18].

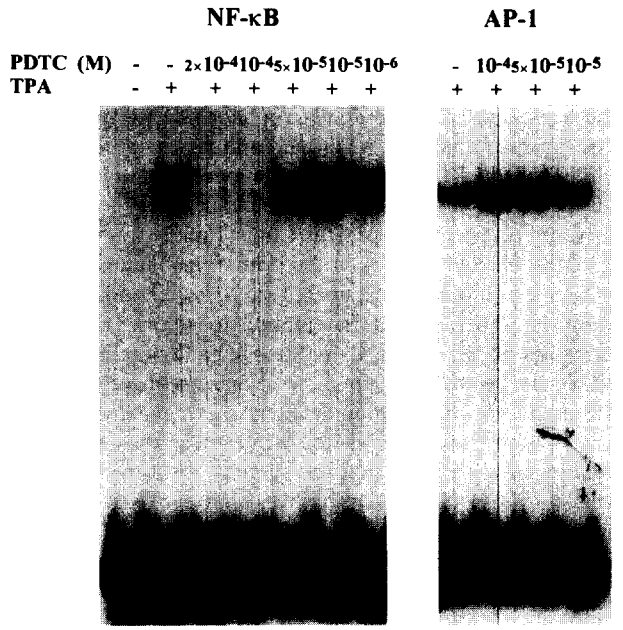

Fig. 6. The ability of PDTC given at 2.0×10^{-4} M or 1.0×10^{-4} M to completely suppress the activation of NF-κB otherwise brought about by TPA (5.0×10^{-8} M) in HeLa cells (left), and the superactivation of the binding of AP-1 to DNA by PDTC at 5.0×10^{-5} M or 1.0×10^{-4} M in the nuclear extracts from the same cells (right).

Although the regulation of the expression of iNOS gene is believed to take place mainly at the transcriptional level [3,12,31,32], few reports have dealt with the actual stability of the transcript of this gene [3]. Previous data from other laboratories suggested that the mechanisms involved in such a control might differ both at the level of the transcription and of the stability of iNOS mRNA according to the cell type (e.g. RAW264.7 cells, mouse peritoneal macrophages, Swiss 3T3 cells) and the primary inducing agent (i.e. LPS, IFN-γ, TGF-β) considered [33–35]. As our findings show, TPA concurrently both super-induced the transcription and shortened the half-life of iNOS mRNA in the primary neonatal rat hepatocytes. Therefore, the extra increase in steady-state levels of iNOS transcript brought about by TPA resulted from a quite remarkable surge in its rate of synthesis, which largely yet temporarily overcame the concurrent notable shortening of the half-life of this same mRNA species also elicited by the phorbol ester.

Unquestionably, TPA did behave in our model as an effective super-inducer of iNOS mRNA transcription even in the presence of a dose of CHX (i.e. 25 μg/ml) capable of completely inhibiting the ongoing hepatocellular protein synthesis. On the other hand, CHX even by itself could similarly operate as a complete and efficient inducer of iNOS gene expression in the primary hepatocytes independently on the dose tested and, hence, on its effects on protein synthesis. In this regard, it has been postulated that CHX can induce the overexpression of specific genes by means of different mechanisms: (i) it hinders at inhibitory concentrations the de novo synthesis of short-lived proteins capable of repressing gene transcription, and/or (ii) it dose-independently and directly favours gene transcription in a TPA-like fashion, via the rapid and sustained phosphorylation of phosphoproteins [36,37]. As to the latter mechanism, CHX given to non-hepatic cells was reported to enhance protein kinase-mediated phosphorylation of cytosolic IκB proteins and, hence, the release and translocation of NF-κB into the nucleus [38]. Interestingly, the activation of NF-κB by some agents (e.g. LPS with or without IFN-γ) has been linked to the subsequent transcription of iNOS mRNA in several types of non-hepatic cells, including macrophage-like RAW264.7 cells, type II airway epithelial A549 cells, and insulin-producing RIN cells [12,27,39]. The present results show that a surge of nuclear NF-κB activity over its controls' baseline levels is a relatively early and transient (i.e. peak at 1 hour rapidly waning thereafter) effect elicited by TPA in the primary neonatal rat hepatocytes, just as it is in other phorbol ester-treated non-hepatic cell types [40]. Therefore, it is tempting to assume that a prior activation of NF-κB be a step necessary for the induction of iNOS gene expression even in the primary neonatal rat hepatocyte model. Moreover, our data make evident that CHX too is by itself an efficient activator of NF-κB in the cultured neonatal hepatocytes, and that the surge of intra-nuclear NF-κB elicited by CHX distinctly outlives that evoked by TPA (cf. lanes 4 and 5 in Fig. 4). Conceivably, TPA and CHX might either activate different nuclear transcription factors or act upon the same transcription factor in the neonatal rat hepatocytes. The latter view seems however to be better suited to explain our findings, as a more persistent activation of NF-κB brought about by the combination of a shorter-lasting effect of TPA with a longer-enduring action of CHX may account for the additive effects exerted on iNOS mRNA transcription by an association of these two agents.

In an endeavour to substantiate the hypothesis that an earlier NF-κB activation be linked to a later iNOS gene expression in the TPA-treated neonatal hepatocytes, we tested the effects of PDTC – a commonly used inhibitor of NF-κB activation in various non-hepatic cell types [13,27,29] – on both these processes. Unexpectedly, PDTC was found to be totally unable to hinder both NF-κB super-activation and iNOS gene hyper-expression in the TPA-treated primary hepatocytes, even though at 10^{-5} M PDTC could simultaneously activate the AP-1 transcription factor in these same cells. This super-activation of AP-1 elicited by a mixture of TPA and PDTC in the primary hepatocytes would suggest that a sufficient amount of the latter agent reached and/or entered the cells in a chemically active form, even though PDTC was not able to prevent the activation of NF-κB by TPA. Conversely, in keeping with Meyer et al. [40], we found that the anti-oxidant PDTC could fully prevent the nuclear translocation of NF-κB elicited by the pro-oxidant TPA in HeLa cells (cf. Fig. 6), in which the TPA-evoked activation of AP-1 was instead enhanced by PDTC. On the whole, these results are consistent with those reported by others in non-hepatic cells [40,42], and indicate that the neonatal hepatocytes' TPA-stimulated protein kinase C makes use of different intracellular signalling pathways in order to activate distinct transcription factors such as NF-κB and AP-1. Such results authorise a warning notice about using PDTC as a general, specific inhibitor of NF-κB activation to invoke the requirement of the latter factor for the expression of certain genes [41]. The likely manifold biological effects of PDTC have not, as yet, been completely clarified. Reportedly, PDTC chelates heavy metals, exerts an anti-oxidant action on account of its dithiocarboxy group, and may break up forming carbon disulphide, a compound reacting with amino groups in proteins (see for references: [41]). As shown by the present results, the effectiveness of PDTC as an inhibitor of NF-κB activation is not universal, but rather is likely to rest upon the specific type of cell considered. Hence, we conclude that TPA activates NF-κB in the primary neonatal rat hepatocytes by means of a signalling pathway that neatly differs from that it uses for the same purpose in HeLa cells.

In conclusion, the present findings show that TPA increases the levels of iNOS mRNA by a transcriptional activation mechanism and that this effect is transient inasmuch as the phorbol ester also shortens the half-life of the same transcript. Moreover, since the inducing effect of TPA does not require new protein synthesis, a directly available transcription factor is likely to be involved. Accordingly, a good candidate factor would be NF-κB, the activation and nuclear translocation of which we found to be enhanced by TPA prior to the induction of iNOS gene transcription in the primary neonatal hepatocytes. However, the existence of a causative relationship between these last two actions of TPA, though likely and appealing as it may seem to be, remains to be proven for the reason that PDTC, the most commonly used inhibitor of NF-κB activation in other types of non-hepatic cells, was found by us to be ineffective under this regard in the cultured rat hepatocytes.

Acknowledgements: This work was supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC, Milan), by the Italian Ministry for University and Scientific Research (MURST, 40% and 60% funds), by the C.N.R. (Target Project on Biotechnology and Bioinstrumentation, Target Project on Ageing, and Italy-Canada Bilateral Project), and by C.I.S.M.I. (Milan, Pavia, Verona).

References

[1] Moncada, S. (1994) J. Hypertens. Suppl. 12, S35–S39.
[2] Bredt, D.S. and Snyder, S.H. (1994) Annu. Rev. Biochem. 63, 175–195.
[3] Morris, S.M., Jr. and Billiar, T.R. (1994) Am. J. Physiol. 266, E829–E839.
[4] Olken, N.M., Osawa, Y. and Marletta, M.A. (1994) Biochemistry 33, 14784–14791.
[5] Harbrecht, B.G. and Billiar, T.R. (1995) Shock 3, 79–87.
[6] Geller, D.A., Nussler, A.K., Di Silvio, M., Lowenstein, C.J., Shapiro, R.A., Wang, S.C., Simmons, R.L. and Billiar, T.R. (1993) Proc. Natl. Acad. Sci. USA 90, 522–526.
[7] Wood, E.R., Berger, H., Jr., Sherman, P.A. and Lapetina, E.G. (1993) Biochem. Biophys. Res. Commun. 191, 767–774.
[8] Helyar, L., Bundschuh, D.S., Laskin, J.D. and Laskin, D.L. (1994) Hepatology 20, 1509–1515.
[9] Geller, D.A., Freeswick, P.D., Nguyen, D., Nussler, A.K., Di Silvio, M., Shapiro, R.A., Wang, S.C., Simmons, R.L. and Billiar, T.R. (1994) Arch. Surg. 129, 165–171.
[10] Evans, C.H. (1995) Agents Actions Suppl. 47, 107–116.
[11] Nunokawa, Y., Ishihara, T., Kanai, T. and Noguchi, T. (1992) Arch. Biochem. Biophys. 298, 726–730.
[12] Lowenstein, C.J., Alley, E.W., Raval, P., Snowman, A.M., Snyder, S.H., Russell, S.W. and Murphy, W.J. (1993) Proc. Natl. Acad. Sci. USA 90, 9730–9734.
[13] Xie, Q.W., Kashiwabara, Y. and Nathan, C. (1994) J. Biol. Chem. 269, 4705–4708.
[14] Adcock, I.M., Brown, C.R., Kwon, O. and Barnes, P.J. (1994) Biochem. Biophys. Res. Commun. 199, 1518–1524.
[15] Kim, H., Lee, H.S., Chang, K.T., Ko, T.H., Baek, K.J. and Kwon, N.S. (1995) J. Immunol. 154, 4741–4748.
[16] Duval, D.L., Sieg, D.J. and Billings, R.E. (1995) Arch. Biochem. Biophys. 316, 699–706.
[17] Itoh, Y., Okanoue, T., Enjo, F., Sakamoto, S., Takami, S., Yasui, K., Kagawa, K. and Kashima, K. (1994) Dig. Dis. Sci. 39, 851–860.
[18] Hortelano, S., Genaro, A.M. and Bosca, L. (1992) J. Biol. Chem. 267, 24937–24940.
[19] Armato, U., Testolin, L., Menegazzi, M., Menapace, L., Ribecco, M., Carcereri dePrati, A., Miwa, M. and Suzuki, H. (1992) Biochem. Biophys. Res. Commun. 182, 1066–1074.
[20] Romano, F., Andreis, P.G., Marchesini, C., Paccagnella, L. and Armato, U. (1986) Toxicol. Pathol. 14, 375–385.
[21] Armato, U., Ribecco, M., Guerriero, C., Testolin, L. and Whitfield, J.F. (1994) J. Cell Physiol. 161, 55–62.
[22] Chirgwin, J.M., Przybyla, A.E., Mac Donald, P.M. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[23] Adachi, H., Iida, S., Oguchi, S., Ohshima, H., Suzuki, H., Nagasaki, K., Kawasaki, H., Sugimura, T. and Esumi, H. (1993) Eur. J. Biochem. 217, 37–43.
[24] Schreiber, E., Mattias, P., Muller, M.M. and Schaffiner, W. (1989) Nucleic Acids Res. 17, 6419
[25] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[26] Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. and Tannenbaum, S.R. (1982) Anal. Biochem. 126, 131–138.
[27] Bedoya, F.J., Flodstrom, M. and Eizirik, D.L. (1995) Biochem. Biophys. Res. Commun. 210, 816–822.
[28] Wang, W.W., Jenkinson, C.P., Griscavage, J.M., Kern, R.M., Arabolos, N.S., Byrns, R.E., Cederbaum, S.D. and Ignarro, L.J. (1995) Biochem. Biophys. Res. Commun. 210, 1009–1016.
[29] Eberhardt, W., Kunz, D. and Pfeilschifter, J. (1994) Biochem. Biophys. Res. Commun. 200, 163–170.
[30] Armato, U., Menegazzi, M., Guerriero, C., Cardinale, C., Carcereri De Prati, A., Tognana, E., Musajo, F. and Suzuki, H. (1995) J. Exp. Clin. Cancer Res. 14, 226–227.
[31] Xie, Q. and Nathan, C. (1994) J. Leukoc. Biol. 56, 576–582.
[32] Nathan, C. and Xie, Q.W. (1994) Cell 78, 915–918.
[33] Gilbert, R.S. and Herschman, H.R. (1993) Biochem. Biophys. Res. Commun. 195, 380–384.
[34] Turco, J. and Winkler, H.H. (1994) Infect. Immun. 62, 2568–2574.
[35] Vodovotz, Y., Bogdan, C., Paik, J., Xie, Q.W. and Nathan, C. (1993) J. Exp. Med. 178, 605–613.
[36] Madahevan, L.C. and Edwards, D.R. (1991) Nature 349, 747–748.
[37] Weisz, A., Oguchi, S., Cicatiello, L. and Esumi, H. (1994) J. Biol. Chem. 269, 8324–8333.
[38] Collart, M.A., Bauerle, P. and Vassali, P. (1990) Mol. Cell Biol. 10, 1498–1506.
[39] Adcock, I.M., Brown, C.R., Kwon, O. and Barnes, P.J. (1994) Biochem. Soc. Trans. 22, 186S
[40] Meyer, M., Scheck, R. and Bauerle, P.A. (1993) EMBO J. 12, 2005–2015.
[41] Moynagh, P.N., Williams, D.C. and O'Neill, L.A.J. (1994) J. Immunol. 153, 2681–2690.
[42] Boyle, W.J., Smeal, T., Defize, L.H.K., Angel, P., Woodgett, J.R., Karin, M. and Hunter, T. (1991) Cell 64, 573–584.