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• *RAPID COMMUNICATION* •

Disrupted NF-κB activation after partial hepatectomy does not impair hepatocyte proliferation in rats

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Abstract

AIM: To analyze the effects of NF-κB inhibition by antioxidant pyrrolidine dithiocarbamate (PDTC) or TNF inhibitor pentoxifylline (PTX) on liver regeneration after partial hepatectomy (PH).

METHODS: Saline, PDTC or PTX were injected 1 h before PH and rats were killed at 0.5 and 24 h after PH. Several control groups were used for comparison (injection control groups).

RESULTS: Compared to saline injected controls, NF-κB activation was absent 0.5 h after PH in rats treated with PDTC or PTX. At 24 h after PH, DNA synthesis and PCNA expression were identical in treated and control rats and thus occurred irrespectively of the status of NF-κB activation at 0.5 h. Signal transducer and activator of transcription 3 (Stat3) activation was observed already 0.5 h after PH in saline, PDTC or PTX group and was similar to Stat3 activation in response to injection without PH.

CONCLUSION: These data strongly suggest that (1) NF-κB p65/p50 DNA binding produced in response to PH is not a signal necessary to initiate the liver regeneration, (2) Stat3 activation is a stress response unrelated to the activation of NF-κB. In conclusion, NF-κB activation is not critically required for the process of liver regeneration after PH.

Key words: Partial hepatectomy; Nuclear factor kappa B; Signal transducer and activator of transcription 3; Hepatocyte proliferation; Antioxidant

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INTRODUCTION

The mechanisms initiating the regenerative response after partial hepatectomy (PH) remain controversial. Several lines of evidence point to the critical role of the activation of nuclear factor kappa B (NF-κB)[1,2]. This is one of the earliest events constantly detectable in the liver after PH and the previous findings have advocated NF-κB being a critical effector of the cascade initiating the regenerative process[3,4]. Some authors also emphasize an anti-apoptotic role of NF-κB during the regenerative process[5]. Mice lacking TNF receptor type 1 (TNF-R1) showed deficient NF-κB binding, low interleukin-6 (IL-6) production, decreased signal transducer and activator of transcription 3 (Stat3) activation and low levels of hepatocyte DNA replication after PH[6-8]. Based on this and similar observations, it has been proposed that activation of the NF-κB pathway is a critical step to usher cells into the cell cycle in response to PH. This process might also require the sequential activation of TNF/NF-κB/IL-6 and Stat3. However, recent studies using mice lacking the common signal transducer of all IL-6 family members gp130 showed only minor effects on the cell cycle and on the peak of DNA synthesis after PH despite an abolished acute phase response and inhibition of Stat3[9]. Moreover, recent studies in a model of transgenic mice, with specific inhibition of NF-κB at the hepatocyte level, did not impair DNA synthesis and did not increase liver apoptosis[10]. In order to clarify the importance of the activation of NF-κB in the liver regeneration process, we have evaluated the consequences of NF-κB inhibition on downstream activation of Stat3 and DNA synthesis after PH. Numerous exogenous and endogenous stimuli are capable of inducing NF-κB activity[11], but the pathways leading to NF-κB activation are complex. One of them involves oxidative stress with elevated level of reactive oxygen species[12,13]. We have therefore administered the anti-oxidant pyrrolidine dithiocarbamate (PDTC), a compound with metal chelator properties that has been used as a reversible inhibitor of NF-κB *in vitro* and *in vivo*[14-17]. Increased production of TNF-α is a potential activator of NF-κB after PH[4], although the source of TNF-α after

PH has not been entirely elucidated. To reduce TNF-α production, we administered pentoxifylline (PTX), a methylxanthine derivative, which has been demonstrated to suppress LPS-induced TNF-α production[18-20]. It may also modulate the expression of other cytokines like IL-6 and IL-1[21,22].

MATERIALS AND METHODS

Animals

Male Wistar rats (220-270 g body weight) were obtained from the Rat Breeding Facilities of the Catholic University of Louvain Medical School, Brussels, Belgium. All animals were kept in a temperature- and humidity-controlled environment in a 12 h light-dark cycle. At all times, they were allowed free access to water and standard food pellet diet (Usine d'Alimentation Rationnelle, Villemoisson-sur-Orges, France). The animals were handled according to the guidelines established by the Catholic University of Louvain.

Surgical procedures and experimental design

All animal manipulations were carried out under light ether anesthesia at room air between 9:00 a.m. and 12:00 noon with the use of a clean, but non-sterile technique. PH consisted in mid-ventral laparotomy and resection of the left lateral and median lobes (70% of the liver), according to Higgins and Anderson[23]. Saline, PDTC (100 mg/kg) and PTX (100 mg/kg) were injected intraperitoneally (i.p.) to the rats 1 h before PH. The rats were killed under ether anesthesia by exsanguination after the puncture of the abdominal aorta and transection of the inferior vena cava in the thoracic cavity, at 0.5 h after PH, i.e. 1 h 30 min after the initial injection of saline or the active compound, and 24 h after PH. Several additional control groups were used for comparison: (1) rats administered saline, PDTC or PTX without PH and killed 1 h after the injection (injection control group), (2) naïve rats that received neither injection not PH (true controls). The livers were removed, lobes were rapidly weighed, snap frozen in liquid nitrogen and stored at -80 ℃. A minimum of three rats was killed in each group at each of the indicated time points.

Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared as previously described[24]. Protein content was determined using a bicinchoninic acid (BCA) protein assay with serum albumin as a standard (Pierce Chemical, Rockford, IL, USA).

Six to ten micrograms of nuclear proteins were pre-incubated for 10 min at room temperature with 2 μg poly (dI-dC) in the following binding buffers: NF-κB (20 mmol/L Hepes, 60 mmol/L KCl, 5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L PMSF, 0.5 mmol/L DTT, glycerol 10%, Nonidet P40 1%); Stat3 (10 mmol/L Hepes, 50 mmol/L NaCl, 1 mmol/L EDTA, glycerol 10%). Double stranded oligonucleotides were ^{32}P end-labeled with γ-^{32}P ATP and added to the extracts (10^5 cpm). The mixtures were incubated for 30 min at room temperature and then electrophoresed (200 V, 2 h) on a 5% polyacrylamide gel in a 1× TBE buffer (25 mmol/L Tris-HCl, 25 mmol/L boric acid, 0.5 mmol/L EDTA). To confirm the identity of the protein/DNA complex, supershift analysis was performed: 4 μL of specific antibody (1 μg/μL) was added to the samples after 30 min of incubation with the labeled probe and incubated for a further 30 min. Polyclonal antibodies against NF-κB components p50 and p65, but also p52, c-rel, and rel-B, and anti-Stat3 were purchased from Santa Cruz (CA, USA). The following probes were used: chromatography-purified double stranded oligonucleotides from the class I major histocompatibility complex enhancer element H2κB; TCGAGGGCTGGGGATTCCC CATCTC (NF-κB) and from the serum-inducible factor binding element in the c-fos promoter; CCAGCATTTCCCGTAAATCCTCCAG; (Stat3). A rabbit reticulocyte (Promega Benelux, Leiden, Netherlands) and an EGF-stimulated A431 cell nuclear extract (Santa Cruz Biotechnology) were used as standards for NF-κB and Stat3, respectively. Gels were dried and exposed to a Kodak Biomax MS film (NEN™ Life Science Products, Inc., Boston, MA, USA) for 16 to 24 h.

Thymidine incorporation

One hour before being killed, 50 μCi of [^{3}H]-Thymidine (Amersham, Buckinghamshire, UK) was administrated into the femoral vein under light ether anesthesia. At the time of killing (24 h after PH), livers were removed, weighed and rapidly snap frozen in liquid nitrogen and stored at -80 ℃. Hepatic DNA synthesis was evaluated by measuring the incorporation of [^{3}H]-Thymidine into the DNA. Total hepatic DNA was extracted as previously described[25], and incorporation of radioactive nucleotide was measured in a liquid scintillation counter (Wallac 1409, Turku, Finland). Its value is expressed as disintegrations per minute (dpm) per μg of DNA. All samples were analyzed in duplicate.

PCNA labeling index

Sections from formalin fixed liver tissue (5 μm thick) were air-dried at 37 ℃ overnight and dewaxed. Slides were incubated for 30 min in H_2O_2 0.3% to inhibit endogenous peroxidases, then in TBS containing 10% normal goat serum (NGS, APP Products Ltd, West Midlands, UK) to block non-specific binding sites. Slides were incubated in a monoclonal primary anti-PCNA mouse antibody (PC10, Dako, Denmark; 1:100; overnight), and after washing, with the secondary antibody (anti-mouse antibody; Boehringer, Mannheim, Germany; 1:500; 30 min) followed by streptavidin peroxidase (Boehringer, Mannheim, Germany; 1:1 000; 30 min). Peroxidase activity was revealed by immersion of the slides for 10 min in a 3,3-diaminobenzidine hypochloride solution (DAB 50 mg/100 mL, pH 7.4, Amersham, Cardiff, UK) supplemented with 0.02% H_2O_2. All slides were treated simultaneously to ensure homogeneity of the technique. PCNA labeling index was obtained by examining 3-5 high-power fields in three rats per group. Each field was divided into four zones,

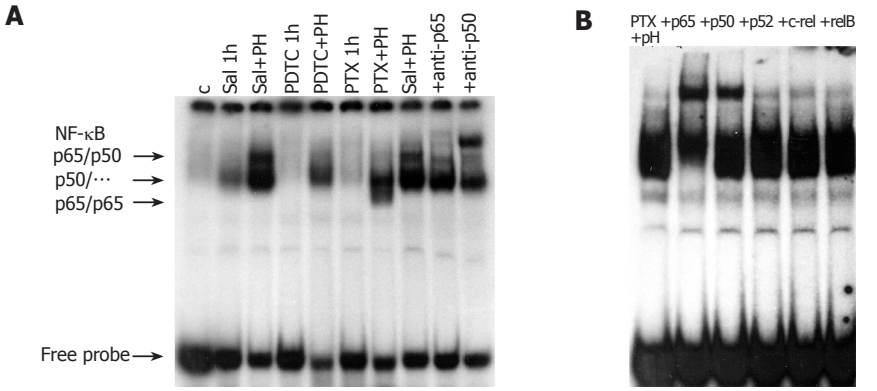

Figure 1 Effect of saline, PDTC, and PTX on NF-κB binding activity. **A:** Nuclear extracts were prepared from non-treated rats (control), from rats injected with saline, PDTC or PTX subjected or not to partial hepatectomy (PH). No NF-κB DNA binding activity was observed in the liver nuclear extracts of non-treated rats (controls) nor 1 h after injection of PDTC or PTX. Faint DNA binding was noticed 1 h after the injection of saline which mainly corresponds to NF-κB p50. Thirty minutes after PH, strong DNA binding was observed in saline-treated groups and two complexes were identified. Supershift experiments identified the upper complex in saline +PH as p65/p50. In the PTX group, a third complex was present 0.5 h after PH, which migrated faster than the dose obtained after saline; **B:** Detailed supershift experiments showed that this faster migrating complex in the PTX+PH group was completely supershifted with the p65 antibody. p52, c-rel and rel-B had no influence on the NF-κB DNA binding activity.

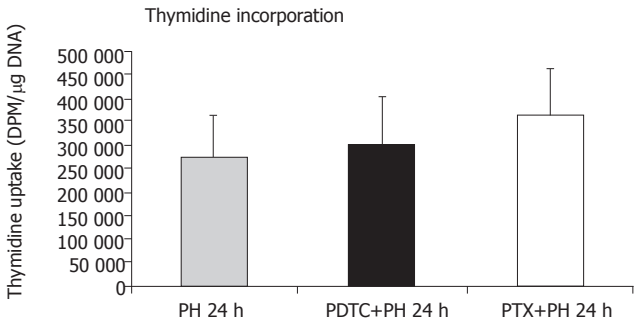

Figure 2 Thymidine incorporation. Similar thymidine incorporation was obtained 24 h after PH in rats pre-treated with an injection of PDTC or PTX compared to non-treated partial hepatectomized rats.

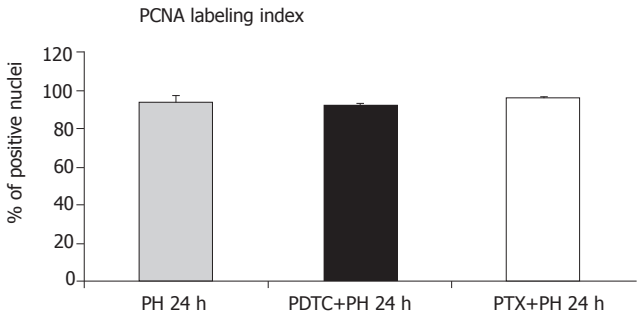

Figure 3 PCNA labeling index. Similar PCNA labeling index was obtained 24 h after PH in rats pre-treated with an injection of PDTC, or PTX compared to non-treated partial hepatectomized rats.

zone 1 being closest to the centrolobular vein and zone 4 corresponding to the periportal areas. Labeling index was defined as the ratio between marked cells and total counted cells.

The same experimented pathologist examined all the slides carefully to detect evidence of necrosis or apoptosis.

Statistical analysis

Results were expressed as mean±SE. The statistical differences between the groups were tested using the one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls multiple comparison tests. Statistical significance was admitted for a P value of <0.05.

RESULTS

Effect of saline, PDTC, and PTX on NF-κB binding activity (Figure 1A)

NF-κB p65/p50 DNA binding activity was not detected in the liver of untreated rats (lane 1) nor in the livers from non-hepatectomized rats 1 h after i.p. injection of saline (lane 2), PDTC (lane 4) or PTX (lane 6). NF-κB was strongly activated 0.5 h after PH preceded by saline injection (lane 3), similarly to activation observed 0.5 h after PH alone[24]. Supershift analysis confirmed the binding of the heterodimer p65/p50 NF-κB complex (lanes 8-10). Injection of PDTC or PTX 1 h before PH prevented the occurrence of the expected p65/p50 DNA binding complex at 0.5 h after PH, since the upper complex was not observed on EMSA (lanes 5 and 7). However, two distinct faster-migrating complexes have been found after PTX (lane 7). The first one, also present after saline or PDTC injection, was partially supershifted by p50 antibody; the second one totally disappeared with p65 antibody. Supershift assays performed with p52, rel B, and c-rel had no influence on this lower complex (Figure 1B).

Effect of PDTC and PTX on hepatocyte proliferation 24 h after PH (Figures 2 and 3)

Hepatocyte proliferation was followed by PH peaks at 24 h. Pretreatment of rats with PDTC or PTX 1 h prior to PH had no effect on liver regeneration. Thymidine incorporation was indeed similarly elevated in all the groups (Figure 2). In untreated rats, 90% of hepatocytes

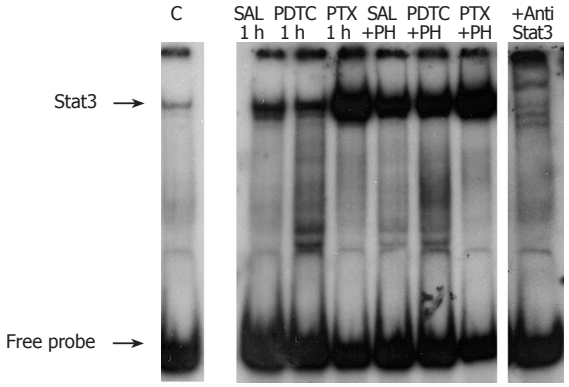

Figure 4 Effect of saline, PDTC and PTX on Stat3 DNA binding activity. No Stat3 DNA binding activity was identified in controls (C). Injection of saline, PDTC or PTX enhanced the binding at 1 h. At 0.5 h after PH, strong Stat3 DNA binding was observed in rats injected with saline, PDTC or PTX. Supershift experiments with a Stat3 specific antibody confirmed that Stat3 is the principal compound of these DNA-binding complexes.

nuclei expressed PCNA 24 h after HP. Pretreatment with PDTC or PTX did not modify the proportion of PCNA positive nuclei in agreement with the thymidine incorporation (Figure 3). No evidence of liver necrosis or massive apoptosis was observed by histological examination.

Effect of injection of saline, PDTC and PTX on Stat3 binding activity (Figure 4)

Stat3 DNA binding activity was not detected in the liver of untreated rats (lane 1), but a Stat3 DNA binding complex was present in the livers from non-hepatectomized rats receiving saline 1 h before being killed (lane 2), PDTC (lane 3) or PTX (lane 4). PH was associated with high Stat3 activity at 0.5 h in animals pretreated with saline (lane 5), PDTC (lane 6) or PTX (lane 7), contrasting with very low Stat3 activation after PH in non-injected rats at this time point. Indeed, as already reported by us and others, PH in non-injected rats was followed by slight Stat3 activation at 0.5 h after PH, peaked at 3 h and was still detected till 8 h[24,26]. Identity of Stat3 was confirmed by supershift analysis with a specific Stat3 antibody (lane 8).

DISCUSSION

PH induces an early cellular response involving pro-inflammatory cytokines like TNF-α or IL-6[27-29] and transcription factors molecules such as NF-κB and Stat3[3,4,30]. In the initiation of proliferating response following hepatic cell mass reduction, a key role seems to be attributed to the activation of the transcription factor NF-κB. The inducible NF-κB factor regulates the expression of numerous genes involved in immune, inflammatory and cellular growth control[11,31,32]. Migrating to the nucleus as soon as 0.5 h after PH[3], it induces the transcription of a large set of immediate-early genes[33]. The NF-κB p65 subunit, important for liver development[34], is responsible for the transcriptional activity of NF-κB[35] and also for its anti-apoptotic effects, preventing the cytotoxic effect of TNF-α and cell death[36,37]. Convergent data suggest that this mediator collaborates with other acute phase gene products in order to protect the cells during the regenerative process[5,38-40]. The most active form of NF-κB is a heterodimer consisting of subunits p50 or NF-κB1 and p65 also called RelA, which contains the transactivation domain necessary for the induction of target genes[41,43]. This active form has been identified after PH as the post-hepatectomy factor[3,30]. Although not consistently reported, the activation of NF-κB after PH has been supposed to be related to an increased expression of TNF-α, although not consistently reported, and/or to oxidative stress[2]. However, various other non specific stimuli are able to induce this activation[44]. Although the importance of the TNF-α pathway has been outlined[6-8], recent studies analyzing liver regeneration in different types of knockout mice suggest that a complete regenerative response may occur in the absence of early factors such as TNF-α, IL-6 or Stat3[43,45]. In order to investigate the role of NF-κB activation in determining the progression into the cell cycle and liver regeneration, we analyzed the effect of NF-κB inhibition on hepatocyte proliferation after PH in normal rat with otherwise normal cytokine expression, regulation and signaling capabilities. Two strategies have been used to prevent NF-κB activation. First, we administered an antioxidant molecule (PDTC) to reduce the oxidative stress, a known stimulus of NF-κB activation. Second, we aimed at decreasing the influence of TNF-α by reducing its production by the use of PTX. PTX and PDTC effectively prevented the activation of NF-κB p65/p50 complex observed 0.5 h after PH in non-treated and saline-treated animals. Despite this absence of NF-κB activation, the hepatocytes responded to the proliferation stimulus: PCNA expression as well as DNA synthesis analyzed 24 h post-PH were normal compared to a classical PH. At that time we did not observe any evidence of liver necrosis or massive apoptosis compared to livers from untreated animals (data not shown). This last observation is in contrast with the results obtained when NF-κB is inhibited by an adenoviral vector expressing a mutated form of IκB-α[5]. The inhibition of NF-κB using this adenoviral vector led to massive apoptosis but also failed to interfere with hepatocyte proliferation. This massive apoptosis could be induced by the adenovirus itself which causes increase of TNF levels and apoptosis before PH. More recently, it has been advocated that the activation of NF-κB in hepatocytes is not needed to induce liver regeneration after PH[10]. The authors also postulated that a preserved activation in non-parenchymal cells could be sufficient to drive both proliferative and anti-apoptotic effects of NF-κB during liver regeneration. This last assumption cannot be found in our experiments, since the inhibition of NF-κB was not targeted to a specific cell type in the liver and, in principal, the substances used in our experiments do act on all liver cell types including the non-parenchymal cells.

We observed that PTX induced another DNA binding

complex after PH, migrating faster, and partially supershifted by the p65 antibody. The functional role of such a complex is not elucidated but may constitute a compensatory response of the Rel family of transcription factors to substitute the usual factor. In a recent study using $p50^{-/-}$ mice[46], PH was associated with a normal regenerative response. P65 protein expression was elevated but without p65/p50 DNA binding activity suggesting that p65 could be part of other complexes, allowing normal regeneration. Considering our data and those obtained in p50 knockout mice, the p65/p50 DNA binding after PH does not seem to be absolutely required for proper liver regeneration. PTX is a potent inhibitor of TNF production which inhibited NF-κB activation but without reducing liver regeneration. These data further reinforce the absence of a link between NF-κB activation and hepatocyte proliferation. However, they do not bring any information concerning a role for TNF-α in the initiation of liver regeneration, since the expression of this factor was not investigated in the study. In contrast to the data demonstrating the quasi absence of Stat3 activation during the first hour after PH[24,26], an early activation of Stat3 was observed as soon as 0.5 h after PH preceded by saline, PDTC and PTX. This suggested that Stat3 was already induced in our model by the first stimulus, i.e. the injection and the required animal handling. This hypothesis was confirmed by the increased Stat3 DNA binding obtained after a single injection of saline, PDTC and PTX. It seems therefore that Stat3 is a component of the acute-phase response induced by stress procedures as minimal as the i.p. injection. This rather non-specific origin for Stat3 activation does not exclude that this factor may play an important role in liver regeneration as documented in studies conducted in IL-6 and TNFR-1 knockout mice in which the restoration of the Stat3 DNA binding following IL-6 supplementation corrected the impaired liver regeneration process[6,47]. It seems thus that Stat3 activation, which is a part of the priming process as described by Bucher *et al*[48], can be produced by a great variety of stimuli and not necessarily by the PH itself.

As demonstrated previously, sham operation also induced activation of NF-κB and Stat3[24]. PH preceded by a sham operation was associated with a second activation of these transcription factors[49]. In this work, the i.p. injection is a minor stress compared to a sham operation. However, the i.p. injection procedure produced by itself an activation of Stat3 that persisted at the time of PH and probably for a longer period, possibly masking the effects induced by this second intervention. By contrast, a NF-κB response was clearly observed after PH when only saline was injected. When drugs able to inhibit NF-κB activation were injected, this response was indeed suppressed but the proliferation indices were not affected. An activation of liver NF-κB seems thus unnecessary to initiate liver regeneration in those conditions. In conclusion, and in complement to those of others using a transgenic model, our data strongly suggest that an increase of liver NF-κB p65/p50 DNA binding as an immediate response to PH is not essential for inducing liver cell proliferation.

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