

# Hypoosmolarity influences the activity of transcription factor NF- $\kappa$ B in rat H4IIE hepatoma cells

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**Abstract** The influence of anisoosmolarity on NF- $\kappa$ B binding activity was studied in H4IIE rat hepatoma cells. Hypoosmolarity induced a sustained NF- $\kappa$ B binding activity whereas the hyperosmotic NF- $\kappa$ B response was only minor. Hypoosmotic NF- $\kappa$ B activation was accompanied by degradation of the inhibitory I $\kappa$ B- $\alpha$ . Protein kinase C, PI<sub>3</sub>-kinase, reactive oxygen intermediates and the proteasome apparently participate in mediating the hypoosmotic effect on NF- $\kappa$ B. Hypoosmolarity plus PMA induced, amplified and prolonged I $\kappa$ B- $\alpha$  degradation and NF- $\kappa$ B binding activity. Transforming growth factor  $\beta$ -induced apoptosis was diminished by hypoosmolarity. However, this anti-apoptotic effect was probably not related to NF- $\kappa$ B activation.

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**Key words:** Anisoosmolarity; NF- $\kappa$ B; Signal transduction; Apoptosis; Hepatoma cell

## 1. Introduction

Alterations of hepatic gene expression and metabolic function occur in response to cell volume alterations under the influence of anisoosmotic environments, hormones, oxidative stress or cumulative substrate uptake [1]. Osmotic regulation of metabolism is a result of an activation of osmosensitive signaling pathways [2].

NF- $\kappa$ B belongs to a family of transcription factors which are activated by many stimuli including inflammatory cytokines, phorbol ester, UV irradiation and reactive oxygen intermediates [3]. The prototypical NF- $\kappa$ B transcription factor is a heterodimer consisting of p50 and p65 (RelA) DNA binding subunits [4]. In unstimulated cells, NF- $\kappa$ B resides in the cytoplasmic space as an inactive complex bound to the inhibitory subunit I $\kappa$ B. Upon stimulation, specific I $\kappa$ B kinases are activated and phosphorylated I $\kappa$ B is degraded by the ubiquitin/proteasome system [5–7]. This releases active NF- $\kappa$ B which translocates to the nucleus and binds to DNA regulatory elements of target genes, such as those for cytokines (IL-1, IL-2, TNF- $\alpha$ ), enzymes (e.g. inducible nitric oxide synthase, inducible cyclooxygenase), adhesion molecules (e.g. ICAM-1, VCAM-1) and receptors (e.g. IL-2 receptor) [8]. The role of NF- $\kappa$ B in regulation of apoptosis is currently controversially discussed [9–11].

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**Abbreviations:** EMSA, electrophoretic mobility shift assay; PARP, poly(ADP-ribose)polymerase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TGF- $\beta$ , transforming growth factor  $\beta$

Here, the activation of NF- $\kappa$ B by hypoosmolarity is demonstrated, which is paralleled by proteolytic I $\kappa$ B- $\alpha$  degradation and which is sensitive to inhibition of protein kinase C (PKC), PI<sub>3</sub>-kinase and to antioxidants. NF- $\kappa$ B is not involved in hypoosmotic protection against transforming growth factor  $\beta$  (TGF- $\beta$ )-induced apoptosis.

## 2. Materials and methods

### 2.1. Chemicals

Cell culture media were from Gibco BRL (Life Technologies GmbH, Karlsruhe, Germany) and fetal bovine serum was from Greiner GmbH (Frickenhausen, Germany). Antibodies against p50, p65 and I $\kappa$ B- $\alpha$  were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MG-132, erbstatin, Gö6850, SB203580, KT5720, PD098059, LY294002, NF- $\kappa$ B SN50, phorbol 12-myristate 13-acetate (PMA) were purchased from Calbiochem-Novabiochem GmbH (Bad Soden, Germany). Sodium orthovanadate, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), glutathione (reduced form) (GSH), *N*-acetyl-L-cysteine (NAC), pyrrolidinedithiocarbamate (PDTC), TGF- $\beta$  were from Sigma (Munich, Germany), Poly(dI-dC) from Pharmacia (Uppsala, Sweden) and [ $\gamma$ -<sup>32</sup>P]ATP from Amersham (Braunschweig, Germany). Protease inhibitor cocktail was purchased from Boehringer (Mannheim, Germany), T4 polynucleotide kinase and oligonucleotides from Promega (Promega GmbH, Mannheim, Germany). All other chemicals were from Merck (Darmstadt, Germany).

### 2.2. Cell culture

H4IIE-C3 rat hepatoma cells were maintained in DMEM/F12 medium supplemented with 10% fetal calf serum (FCS) in a 37°C, 5% CO<sub>2</sub> atmosphere. When cells reached 90% confluence, the medium was changed to DMEM/F12 without FCS for 24 h. Extracellular osmolarity was adjusted by dilution of the media with the appropriate volume of the respective NaCl-free medium leading to hypoosmolarity (205 mosmol/l) or with medium of elevated NaCl content leading to hyperosmolarity (405 mosmol/l), respectively. Cells were sensitized with 500  $\mu$ M sodium vanadate during experimental treatment if not indicated otherwise.

### 2.3. Preparation of cytosolic and nuclear extracts

The cells were washed twice with ice cold phosphate-buffered saline (PBS). After adding buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT, 1 mM sodium vanadate, protease inhibitors), cells were kept on ice for 10 min. Cells were harvested and nuclei were collected through centrifugation at 10000 $\times$ g. The cytosolic supernatant was used for I $\kappa$ B- $\alpha$  Western blot analysis. The nuclei were resolved in buffer B (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 1 mM sodium vanadate, protease inhibitors). After an incubation period of 20 min on ice, nuclear extract was collected through centrifugation at 3500 $\times$ g. The protein concentration of the nuclear supernatant was measured according to the manufacturer's instruction (Bio-Rad).

### 2.4. Electrophoretic mobility shift assay (EMSA)

A double-stranded oligonucleotide containing NF- $\kappa$ B binding site (5'-AGTTGAGGGGACTTTCACAGGC-3') was end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase. The labeled probes were purified from free nucleotides using Qiagen Nucleotide Removal kit.

NF- $\kappa$ B DNA binding reactions were performed in a 30  $\mu$ l volume containing 4–5  $\mu$ g of nuclear extract, 6  $\mu$ l 5 $\times$  binding buffer (20 mM HEPES pH 7.9, 60 mM KCl, 2 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 10 mM DTT, 5% glycerol), 0.3  $\mu$ g of poly(dI-dC) and the labeled oligonucleotide. Reactions were incubated at room temperature for 30 min and analyzed by electrophoresis on a 6% non-denaturing polyacrylamide gel, 0.5 $\times$ TBE (50 mM Tris, 50 mM boric acid, 2 mM EDTA) pH 8.2. Gels were dried and DNA-protein binding complex was localized by autoradiography for 12 h. For supershift analysis, 0.5  $\mu$ g of p50 or p65 antibody was added.

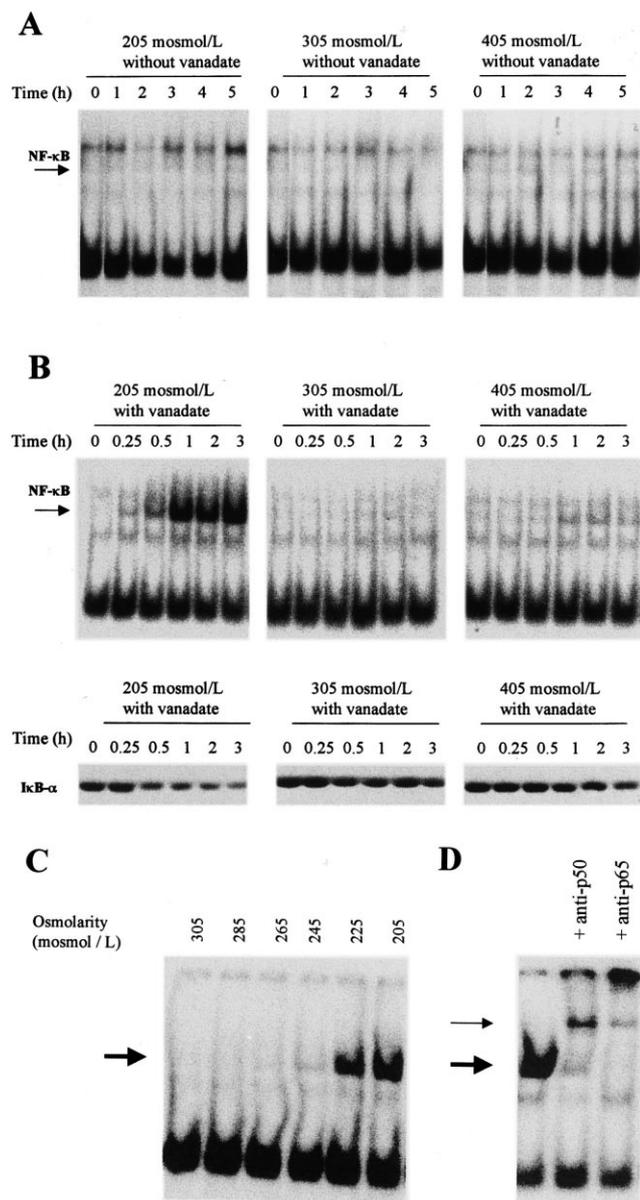


Fig. 1. Induction of NF- $\kappa$ B binding activity and corresponding I $\kappa$ B- $\alpha$  degradation. (A) Cells were incubated in hypoosmotic (205 mosmol/l), normoosmotic (305 mosmol/l) or hyperosmotic (405 mosmol/l) media without (A) or with (B) orthovanadate for the indicated time periods. Nuclear extracts were used for gel shift assay. Arrow shows the inducible complex. Cytosolic supernatants were analyzed in I $\kappa$ B- $\alpha$  immunoblot assay (B). NF- $\kappa$ B binding activity measured after 3 h in an anisoosmolar medium ranging from 305 to 205 mosmol/l (C). Cells were exposed to 205 mosmol/l for 2.5 h. Inducible complex was identified as p50/p65 heterodimer using supershift analysis with specific p50 and p65 antibodies. Thick arrow indicates the inducible complex. Thin arrow shows supershift (D).

## 2.5. I $\kappa$ B- $\alpha$ and poly(ADP-ribose)polymerase (PARP) immunoblot analysis

For I $\kappa$ B- $\alpha$  immunoblot analysis, 20  $\mu$ g of the cytosolic supernatant was resolved on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and electrotransferred to nitrocellulose filters. Non-specific binding sites were blocked with Tris-buffered saline (TBS) with 0.1% Tween 20 (TBST) containing 5% bovine serum albumin (BSA). Membranes were incubated in a 1:1000 dilution of rabbit polyclonal anti-I $\kappa$ B- $\alpha$  in TBST 2% BSA. After three washes in TBST, membranes were incubated in a 1:10 000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG. Immunoreactive proteins were detected by enhanced chemiluminescence. For PARP immunoblot analysis, cells were lysed in RIPA (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors) and resolved on 8% SDS-polyacrylamide gels. PARP cleavage was detected by an anti-PARP antibody (Pharmingen).

## 2.6. Caspase-3 activity

Cells were treated with 5 ng/ml TGF- $\beta$  for 12 h, washed twice with PBS, harvested with trypsin and 2 million cells for each reaction were used. The activity of caspase-3 was determined by using Clontech colorimetric assay kit according to the manufacturer's instructions.

## 3. Results

### 3.1. Hypoosmotic activation of NF- $\kappa$ B

Activation of NF- $\kappa$ B transcription factor following hypoosmotic exposure was shown by EMSA and I $\kappa$ B- $\alpha$  Western blot analysis. Cells were starved for 12 h prior to exposure to hypoosmotic (205 mosmol/l), normoosmotic (305 mosmol/l) and hyperosmotic (405 mosmol/l) medium without (Fig. 1A) or with sodium orthovanadate at a final concentration of 500  $\mu$ M (Fig. 1B). Nuclear and cytosolic extracts were prepared as described and analyzed. Hypoosmotic exposure of orthovanadate-sensitized cells was followed by a strong and persistent increase of binding activity. The effect on hyperosmolarity was only slight. No NF- $\kappa$ B activation could be detected under normoosmotic conditions (Fig. 1B), or in absence of orthovanadate. In agreement with the hypoosmotic-induced increase of NF- $\kappa$ B binding activity in nucleic extracts, a reduction of inhibitory subunit I $\kappa$ B- $\alpha$  was observed in the corresponding cytosolic fractions (Fig. 1B). NF- $\kappa$ B activation was osmolarity-dependent and already appeared at 245 mosmol/l (Fig. 1C). The inducible complex in the gel shift assay could be identified as the prototypical p50/p65 heterodimer using supershift analysis (Fig. 1D).

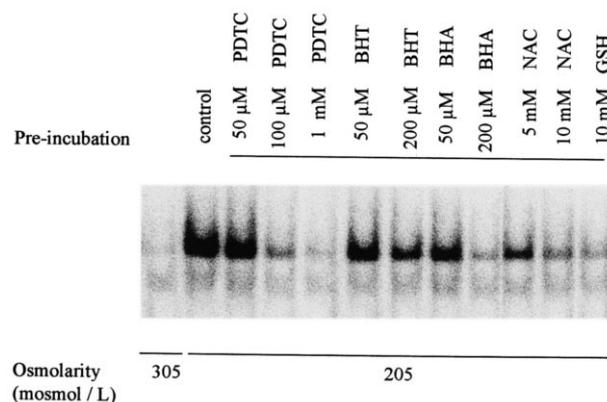


Fig. 2. Effect of antioxidants on NF- $\kappa$ B activation. Cells were treated with antioxidants at the concentrations indicated for 30 min prior to hypoosmotic exposure. Cells were harvested and EMSA was performed as described.

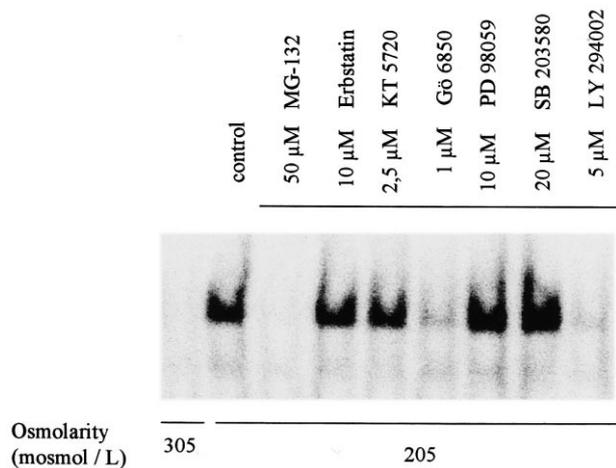


Fig. 3. Sensitivity of NF- $\kappa$ B binding activity to proteasome and kinase inhibitors. Cells were treated with inhibitors as indicated 30 min prior to hypoosmotic exposure. Nucleic extracts were analyzed using gel shift assay.

### 3.2. Inhibition of the hypoosmotically induced NF- $\kappa$ B binding activity by antioxidants

Oxidative stress is a known inducer of NF- $\kappa$ B. Therefore, the influence of antioxidants was examined. As shown in Fig. 2, several antioxidants were able to reduce NF- $\kappa$ B binding activity after hypoosmotic exposure. These antioxidants included chelators (PDTC), synthetic aromatic compounds (BHT, BHA) and sulfhydryl group donors (NAC and GSH).

### 3.3. Pharmacological characterization of hypoosmotic-induced NF- $\kappa$ B binding

As shown in Fig. 3, hypoosmotic NF- $\kappa$ B activation was sensitive to inhibition of PKC by Gö6850 [12] and inhibition of PI<sub>3</sub>-kinase by LY294002 [13]. The reversible proteasome inhibitor MG-132 [14] decreased transcription factor binding activity to baseline levels, suggesting an involvement of proteasomes. Protein tyrosine kinases, protein kinase A, MAP kinase and p38<sup>MAPK</sup> as kinases with central importance in signal transduction are apparently not involved in NF- $\kappa$ B

transcription factor regulation as suggested by the lack of an effect of erbstatin [15], KT5720 [16], PD98059 [17] and SB203580 [18].

### 3.4. Modulation of PMA-induced NF- $\kappa$ B activity by hypoosmolarity

Fig. 4 shows the influence of the osmolarity on PMA-induced NF- $\kappa$ B activation. Under normoosmotic conditions, NF- $\kappa$ B is rapidly activated and translocates to the nucleus within 1 h of PMA addition. Corresponding to this NF- $\kappa$ B activation and translocation, a degradation of I $\kappa$ B- $\alpha$  could be observed. This activation was transient as was the decrease of I $\kappa$ B- $\alpha$ . The NF- $\kappa$ B binding activity returned to baseline within 6 h.

On the other hand, hypoosmotic-induced NF- $\kappa$ B activation showed a slower increase of NF- $\kappa$ B binding activity which was also accompanied by I $\kappa$ B- $\alpha$  degradation. When cells were treated with hypoosmolarity plus PMA, a long lasting strong NF- $\kappa$ B binding activity was observed and I $\kappa$ B- $\alpha$  was downregulated over 6 h.

### 3.5. Effect of hypoosmolarity on TGF- $\beta$ -induced caspase-3 activity and PARP cleavage

The influence of hypoosmotic exposure on cell survival was measured using the caspase-3 colorimetric assay (Fig. 5). Whereas caspase-3 activity under normoosmotic and hypoosmotic conditions showed comparable values, addition of TGF- $\beta$  to a final concentration of 5 ng/ml in normoosmotic medium caused a significant increase of caspase-3 activity. This increase was prevented by decreasing osmolarity to 205 mosmol/l 30 min prior to TGF- $\beta$  addition. Neither PDTC (1 mM), which inhibits hypoosmotic NF- $\kappa$ B activation (Fig. 5), nor SN50, a specific NF- $\kappa$ B inhibitor [19], had significant effects on the hypoosmotic inhibition of TGF- $\beta$ -induced activation of caspase-3 activity. These findings suggest that NF- $\kappa$ B is probably not involved in mediating the inhibitory effect of hypoosmolarity on the TGF- $\beta$ -induced caspase-3 activation. In agreement, PARP cleavage appeared only after TGF- $\beta$  treatment under normoosmotic conditions (Fig. 5), but was suppressed by hypoosmolarity regardless of whether hypoosmotic NF- $\kappa$ B activation was inhibited by PDTC or not.

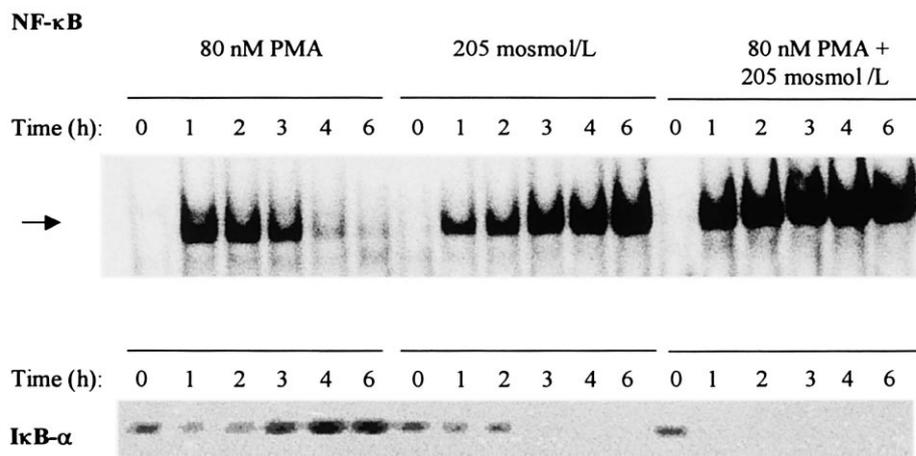


Fig. 4. Influence of osmolarity on NF- $\kappa$ B binding activity and I $\kappa$ B- $\alpha$  degradation following PKC stimulation. Measurement of NF- $\kappa$ B binding activity after stimulation of H4IE cells with PMA in normoosmotic medium (305 mosmol/l) or in hypoosmotic medium (205 mosmol/l). Immunoblot analysis of I $\kappa$ B- $\alpha$  of corresponding cytosolic supernatant.

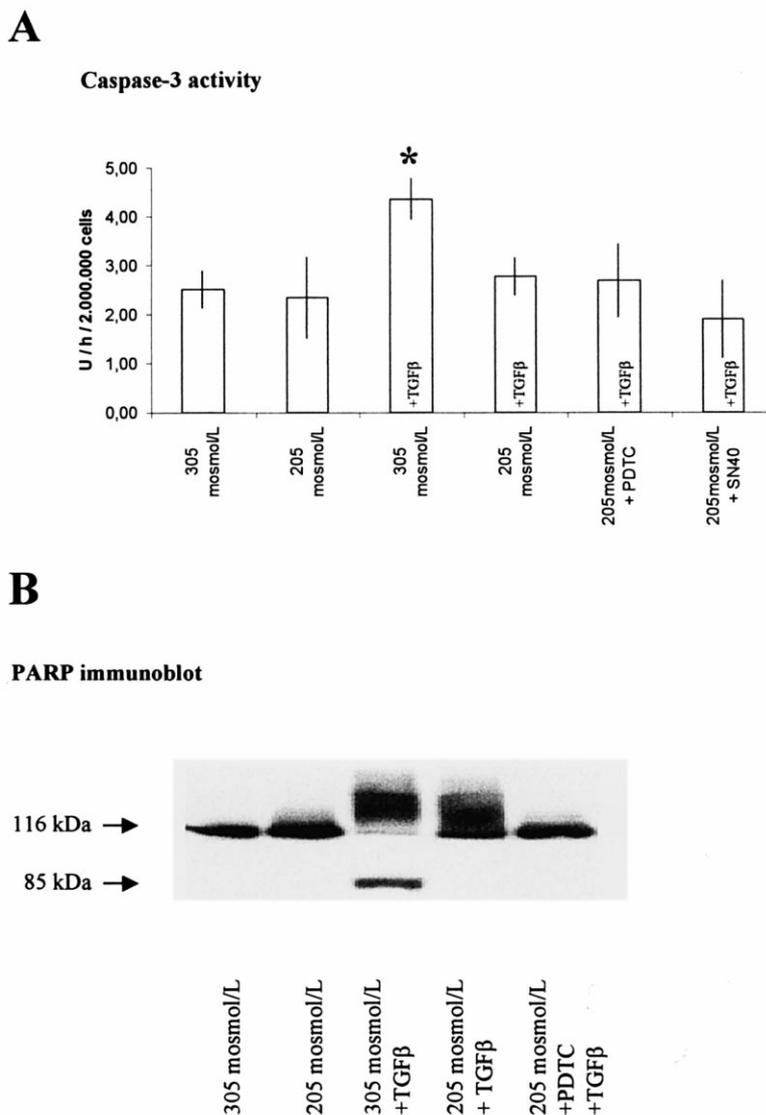


Fig. 5. Effect of osmolarity on caspase-3 activity and PARP cleavage of TGF- $\beta$ -treated cells. Cells were exposed to normoosmotic or hypoosmotic media as indicated for 30 min. TGF- $\beta$  was added to a final concentration of 5 ng/ml and incubation was continued for a period of 12 h (caspase-3) or 8 h (PARP). Cells were harvested as described. For NF- $\kappa$ B inhibition, PDTC (1 mM) and specific translocation inhibitor NF- $\kappa$ B SN50 (20  $\mu$ M) were added 30 min prior to anisoosmolar stimulation. Caspase-3 activity was measured by colorimetric assay. PARP cleavage was visualized by immunoblot analysis.

#### 4. Discussion

In the present paper, the hypoosmotic activation of NF- $\kappa$ B binding towards its specific DNA sequence is reported, which is accompanied by degradation of I $\kappa$ B- $\alpha$  and which apparently depends on reactive oxygen intermediates (Fig. 2), PKC and PI $_3$ -kinase (Fig. 3). PMA amplifies the hypoosmotic effect on NF- $\kappa$ B activity and I $\kappa$ B- $\alpha$  degradation (Fig. 4). NF- $\kappa$ B is apparently not involved in mediating the anti-apoptotic effect of hypoosmolarity on TGF- $\beta$ -treated H4IIE cells (Fig. 5).

The upstream signalling components mediating hypoosmotic NF- $\kappa$ B activation differ from those leading to hypoosmotic stimulation of the MAP kinases Erk-1/Erk-2 in H4IIE cells and hepatocytes, which were sensitive to genistein but resistant to inhibition of PKC [20,21]. Accordingly, NF- $\kappa$ B activation remained unaffected in the presence of PD098059

(Fig. 3), which inhibits the MAP kinase kinase immediately upstream of Erk-1/Erk-2. Also the hypoosmotic activation of the p38<sup>MAPK</sup> observed in H4IIE cells [22] and hepatocytes [23] is apparently not involved (Fig. 3). A participation of hypoosmolarity-induced JNK activation [24] in NF- $\kappa$ B activation cannot be excluded, although divergent pathways lead to JNKs and NF- $\kappa$ B in the case of TNF- $\alpha$ -stimulated MCF7 and HeLa cells [25]. Hypoosmolarity activates PI $_3$ -kinase and PKC in hepatocytes [26,27]. In Jurkat cells, PI $_3$ -kinase was recently demonstrated to mediate NF- $\kappa$ B activation upstream of I $\kappa$ B- $\alpha$  via activation of the Akt/PKB kinase and the effect of Akt is most striking if cells are costimulated with PMA [28]. This would favor the view that PKC and PI $_3$ -kinase represent two signalling branches activated by hypoosmolarity, which converge upstream of I $\kappa$ B- $\alpha$  degradation in H4IIE cells. PMA is known to stimulate the generation of reactive oxygen intermediates in NIH3T3 cells [29], point-

ing to a potential role of PKC in redox signalling towards NF- $\kappa$ B. PMA induces a transient NF- $\kappa$ B activation in H4IIE cells, accompanied by I $\kappa$ B- $\alpha$  degradation and followed by resynthesis of I $\kappa$ B- $\alpha$  (Fig. 4), which probably induces downregulation of NF- $\kappa$ B binding activity within a feedback loop. The reason for interruption of feedback inhibition under hypoosmotic conditions (Fig. 4) is yet unknown, but may contribute to sustained NF- $\kappa$ B activation observed under certain conditions [30].

Hypoosmotic cell swelling downregulates autophagic proteolysis in the liver [23]. Contrasting to the effect on overall hepatic proteolysis, hypoosmolarity stimulates the degradation of I $\kappa$ B- $\alpha$  (Figs. 1 and 4), which is known to occur via the ubiquitin/proteasome pathway. Obviously, hypoosmolarity exerts differential effects on the degradation rate of single proteins. To what extent the proteasome pathway underlies regulation by cell hydration remains to be determined.

Conflicting data exist with respect to the relation of NF- $\kappa$ B to the apoptotic process. In HEK 293 cells, activation of NF- $\kappa$ B was suggested to be involved in mediating apoptosis [31]. In contrast, NF- $\kappa$ B suppressed TNF- $\alpha$ -induced apoptosis in HEF cells [32]. Insulin, which activates NF- $\kappa$ B and PI $_3$ -kinase via different signalling branches, exerts its anti-apoptotic function by both a NF- $\kappa$ B- and a PI $_3$ -kinase-dependent pathway [33]. In line with the protective effect of hypoosmotic swelling on cells challenged with oxidative stress or heat shock [24,34–36], hypoosmolarity abolishes TGF- $\beta$ -induced activation of caspase-3 and PARP cleavage (Fig. 5). However, blockade of NF- $\kappa$ B activation by the antioxidant PDTC is without influence on the anti-apoptotic effect exerted by hypoosmotic swelling (Fig. 5). One can speculate that signalling components different from NF- $\kappa$ B such as PI $_3$ -kinase mediate the anti-apoptotic effect of hypoosmolarity. Alternatively, hypoosmotic swelling may interfere with cell shrinkage, which is an obligatory event during the apoptotic cell death.

Although the functional importance of hypoosmotic NF- $\kappa$ B activation in liver remains to be established, it likely contributes to osmotic regulation of hepatic gene expression and modulation of signalling induced by hormones and stress conditions.

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