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Signaling pathway underlying the up-regulatory effect of TNF-α on the Na^+/K^+ ATPase in HepG2 cells

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ABSTRACT

The activity of the Na^+/K^+ ATPase was shown to be reduced during apoptosis and enhanced during cell proliferation. This work investigated whether TNF-α exerts also opposite effects on the Na^+/K^+ ATPase in HepG2 cells and whether these effects are time-dependent. A time response study demonstrated that the activity and protein expression of the ATPase are decreased at 1 h and increased at 4, 6 and 8 h. This work focused on the up-regulatory 4 h-response. TNF-α was shown to exert a stimulatory effect on cJNK and NF-κB and an inhibitory effect on caspases which, in the basal state, down-regulate the ATPase. The cytokine was found to target the caspases by activating JNK which in turn activates NF-κB. The activated transcription factor inhibits the caspases and frees the ATPase from their inhibitory action leading thus to its up-regulation.

1. Introduction

Tumor necrosis factor α (TNF-α) is a pro-inflammatory cytokine with numerous biological properties ranging from inflammation to proliferation, differentiation, and cancer growth. It plays a central role in shock due to sepsis and wasting syndromes due to a variety of cancers [1]. It is also involved in different physiological and pathophysiological conditions [2]. In the liver it was described to act as a "two-edged sword" [3], exerting both apoptotic and anti-apoptotic effects.

These antagonistic effects were ascribed to the multiple and complex signaling pathways activated by the cytokine. Caspases, nuclear factor kappa B (NF-κB), Jun N-terminal kinase (JNK) [4], proteases, ceramides, and reactive oxygen species, were all found to mediate TNF-α action. While activation of NF-κB was found crucial for cell survival, activation of caspases was deemed essential for the apoptotic process. The role of JNK is however still controversial promoting both apoptotic and anti-apoptotic responses [5].

Apoptosis was found to be accompanied by changes in the levels of Na^+, K^+, and Cl^-, and a decrease in the activity of the Na^+/K^+ ATPase also known as Na^+/K^+ pump [6], a ubiquitous transporter responsible for the establishment of the trans-membrane Na^+ and K^+ gradients. In a previous work we studied the 1 h effect of TNF-α on the Na^+-/K^+ ATPase in HepG2 cells. The activity of the ATPase was found to be modulated by JNK, which stimulated the caspases and inhibited NF-κB, resulting in a net inhibition of the pump, and probably favoring the apoptotic pathway [7]. Since TNF was reported also to promote cell proliferation in hepatocytes [8], we speculated that these antagonistic responses may be due to differences in the period of action of the cytokine. TNF-α may be activating different mediators at different time intervals leading to opposite effects.

This work aimed at testing this hypothesis and determining the mechanism of action of the cytokine.

2. Materials and methods

2.1. Materials

Media and other chemicals needed for cell culture were purchased from Gibco, Paisley, Scotland.

The general caspase inhibitor (FK009) and the JNK inhibitor (SP 600125) were purchased, respectively, from MP Biomedicals, Ohio, USA, and Biosource, CA, USA.

Recombinant Human TNF alpha was purchased from Endogen, MA, USA. The primary Na^+/K^+ ATPase α1 antibody was obtained from Upstate Va., USA, and the HepG2 cell line from ATCC.

Anti-IκBα monoclonal antibody was obtained from Biosource International, USA. Goat anti-mouse IgG HPR conjugated antibody and Enhanced Chemiluminescence (ECL) kit were obtained from Santa Cruz, CA, USA.

JNK activity was measured using a KinasesSTAR™ JNK Activity Assay Kit purchased from BioVision CA, USA.

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All other chemicals were obtained from Sigma Chemical Co., St. Louis, Missouri, USA.

2.2. Methods

2.2.1. Culture and treatment of HepG2 Cells

HepG2 cells were grown in DMEM supplemented with 10% FBS, 1% Penicillin and streptomycin, in a humidified incubator (5% CO2) at 37 °C. When 70–80% confluence was reached, the cells were starved overnight and treated next day.

Dose and time response studies were conducted by treating the cells with different doses of TNF-α (5, 10, 40, and 100 ng/ml) for 4 h, and with 100 ng/ml of TNF-α for different time periods (1, 2, 3, 4, 6, and 8 h). The involvement of caspases, JNK and NF-κB was studied by pre-treating the cells with their respective specific inhibitors FK009 (4 μM) ([9], SP 600125 (50 μM) [10] and PDTC (100 μM) [11] . FK009 was added 15 min before TNF-α, while PDTC and SP 600125 were added 30 min before the cytokine.

At the end of the incubation period, HepG2 cells were washed, lysed, homogenized with a polytron at 4 °C and 20,000 rpm, and preserved at −20 °C for later use after addition of protease inhibitors. Proteins were quantified using the Biorad assay.

2.2.2. Western blot analysis

Equal amounts of protein were loaded and resolved on 8% polyacrylamide gels, and were transferred to PVD F membranes. The membranes were then blocked and incubated with a primary Na^+/K^+ ATPase α1 antibody, followed by a secondary IgG conjugated to horseradish peroxidase. Detection of the signal was by enhanced chemiluminescence. Equal loading was checked by Ponceau staining, a rapid and reversible staining method for visualizing protein bands on Western blots.

2.2.3. NF-κB activity

Activation of NF-κB by TNF was investigated by examining the degradation of its inhibitory subunit IκBα. The expression of IκB was studied by western blot analysis as described above using 10% polyacrylamide gels and an anti-IκBα monoclonal antibody with a goat anti-mouse secondary IgG conjugated to horseradish peroxidase.

2.2.4. Na^+/K^+ ATPase activity assay

Homogenates of HepG2 cells were suspended in Tris buffer (1300 mM NaCl, 200 mM KCl, 40 mM $MgCl_2$ and 150 mM histidine; pH 7.4) to a concentration of 2 mg/ml. Saponin (1%) was then added at a ratio of 1:4 and the samples were incubated at room temperature for 30 min. Aliquots (50 μl) were then drawn and incubated at 37 °C for 30 min, in presence or absence of ouabain (3.75 mM final concentration), a specific inhibitor of the ATPase. Adenosine tri-phosphate (ATP) (7.5 mM final concentration) was then added and the samples were incubated for an additional 30 min at 37 °C. At the end of the incubation period, an equal volume of trichloroacetic acid (11.5%) was added to stop the reaction, and the samples were spun at 3000g for 5 min. The amount of inorganic phosphate liberated was measured colorimetrically according to the method of Taussky and Shorr [12]. The enzymatic activity was determined by measuring the ouabain inhibitable inorganic phosphate (Pi) released. The percent inhibition of the enzyme activity was calculated as follows:

$$1 - \frac{P_i(treatment) - P_i(treatment + ouabain)}{P_i(control) - P_i(control + ouabain)} \times 100$$

2.2.5. c-Jun N-terminal kinase activity

A "KinasesStar JNK activity assay kit" was used to measure JNK activity according to the manufacturer's instructions. Briefly JNK was immunoprecipitated from cell lysates and its activity determined using recombinant c_jun as a substrate. Phosphorylation of c-Jun was detected by western blot analysis.

2.2.6. Statistical analysis

Results are reported as means ± SE and are tested for statistical significance by a one-way Analysis of Variance (ANOVA) followed by Tukey–Kramer multiple comparisons test using Instat and Excel Analysis softwares. The results were considered significant at $P < 0.01$.

3. Results

3.1. Time and dose response studies on the effect of TNF-α on the Na^+/K^+ ATPase in HepG2 cells

TNF-α at 100 ng/ml reduced the activity and protein expression of the Na^+/K^+ ATPase at 1 h, and enhanced them at 4, 6 and 8 h (Fig. 1a). The most prominent increase was observed at 4 h and was maintained till 8 h ($P < 0.001$). This is why all further studies were conducted at 4 h. The effect on pump's expression followed the same pattern as the effect on the pump's activity (Fig. 1b).

A dose response study was then conducted to see if the effect of the cytokine on the pump is concentration-dependent. The activity of the Na^+/K^+ATPase was significantly increased in starved cells treated for 4 h with 40 and 100 ng/ml TNF-α and unchanged at lower doses (5 and 10 ng/ml) (Fig. 1c).

Since the up-regulatory effect observed at 4 h opposed what was found previously at 1 h, we decided to investigate the 4-h response and the signaling pathways involved.

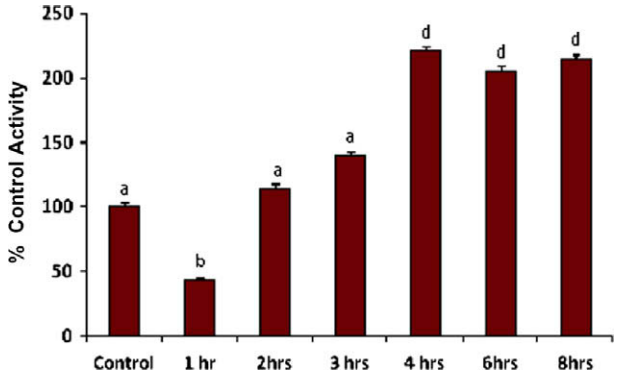

Fig. 1a. Time response study on the effect of TNF-α on the activity of the Na^+/K^+ ATPase in HepG2 cells. Values are means ± SE of three observations assayed each in triplicates. Bars not sharing a common letter are significantly different from each other. $P < 0.001$.

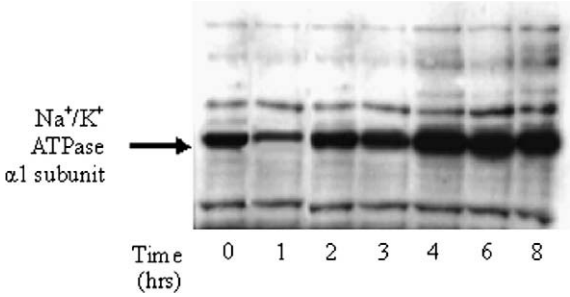

Fig. 1b. Time response study on the effect of TNF-α on the protein expression of the Na^+/K^+ ATPase in HepG2 cells. The results are representative of an experiment repeated three times.

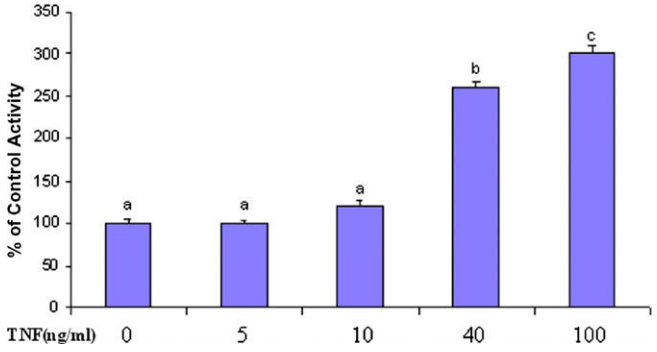

Fig. 1c. Dose response study on the effect of TNF-α on the activity of Na^+/K^+ ATPase in HepG2 cells treated with the cytokine for 4 h. Values are means ± SE of three observations assayed each in triplicates. Bars not sharing a common letter are significantly different from each other. $P < 0.01$.

3.3. Mediators of the late (4 h) effect of TNF-α on the Na^+/K^+ ATPase in HepG2 cells

3.3.1. Involvement of JNK and NF-KB

At 4 h, TNF decreased the expression of IKBα (Fig. 2a) and increased significantly the phosphorylation of c-jun (Fig. 2b) revealing an activation of NF-κB and JNK by the cytokine.

The involvement of NF-κB and Jun Kinase in the late (4 h) response was studied by using their respective inhibitors PDTC and SP600125.

PDTC alone had no effect on the expression and activity of the pump but abrogated completely the effect of TNF-α (Fig. 3a and b). The ATPase was down-regulated by SP600125 and in its presence the up-regulatory effect of TNF was significantly reduced (Fig. 3a and b).

3.3.2. Involvement of caspases

To determine whether TNF-α signaling is mediated through caspases, HepG2 cells were treated with FK009 a general caspase inhibitor. The inhibitor FK009 alone, increased both the activity and protein expression of the pump, exerting thus a similar effect to the one observed with TNF-α (Fig. 4a and b). The up-regulatory effect of TNF still appeared in presence of FK009 i.e. when caspases were inhibited.

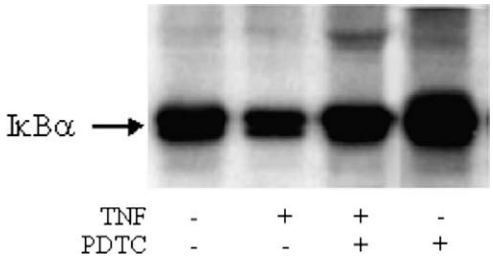

Fig. 2a. Effect of TNF-α (100 ng/ml, 4 h) on the expression of IκBα.

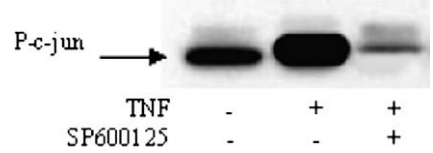

Fig. 2b. Effect of TNF-α (100 ng/ml, 4 h) on the phosphorylation of c-jun.

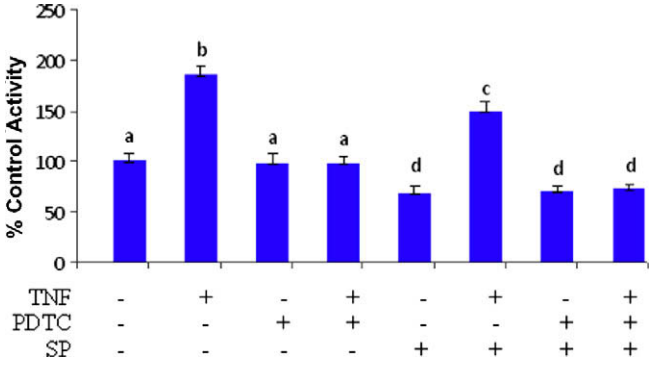

Fig. 3a. Effect of TNF-α, in presence and absence of PDTC and SP600125, on the activity of the Na^+/K^+ ATPase in HepG2 cells treated with the cytokine for 4 h. Values are means ± SE of three observations assayed each in triplicates. Bars not sharing a common letter are significantly different from each other. $P < 0.01$.

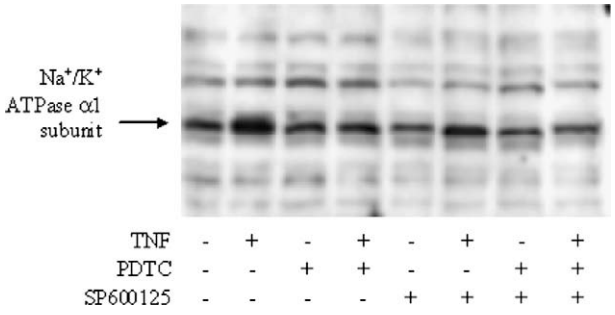

Fig. 3b. Effect of TNF-α, in presence and absence of PDTC and SP600125, on the activity of the Na^+/K^+ ATPase in HepG2 cells treated with the cytokine for 4 h. The results are representative of an experiment repeated three times.

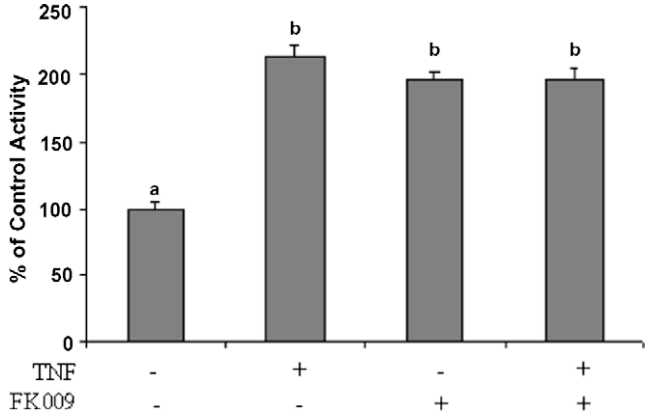

Fig. 4a. Effect of TNF-α in presence and absence of FK009 on the activity of the Na^+/K^+ ATPase in HepG2 cells incubated with the cytokine for 4 h. Values are means ± SE of three observations assayed each in triplicates. Bars not sharing a common letter are significantly different from each other. $P < 0.01$.

3.3.3. Effect of different combinations of inhibitors on the protein expression and activity of the Na^+/K^+ ATPase at 4 h

When both jun Kinase and NF-κB were inhibited concurrently with their respective inhibitors SP600125 and PDTC, a decrease in the activity and protein expression of the Na^+/K^+ ATPase was observed (Fig. 3a and b). This decrease still appeared in presence of the cytokine.

The up-regulatory effect exerted by FK009 was reduced in presence of PDTC (Fig. 5a and b), but in their simultaneous presence the

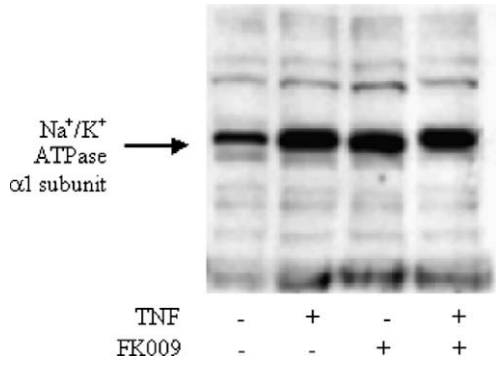

Fig. 4b. Effect of TNF-α in presence and absence of FK009 on the protein expression of the Na^+/K^+ ATPase in HepG2 cells incubated with the cytokine for 4 h. The results are representative of an experiment repeated three times.

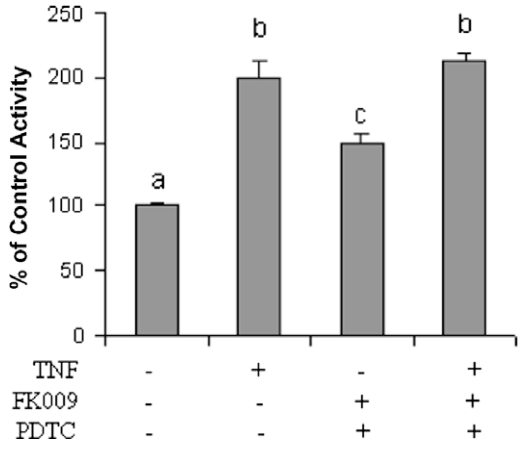

Fig. 5a. Effect of TNF-α in the simultaneous presence and absence of FK009 and PDTC on the activity of the Na^+/K^+ ATPase in HepG2 cells incubated with the cytokine for 4 h. Values are means ± SE of three observations assayed each in triplicates. Bars not sharing a common letter are significantly different from each other. $P < 0.01$.

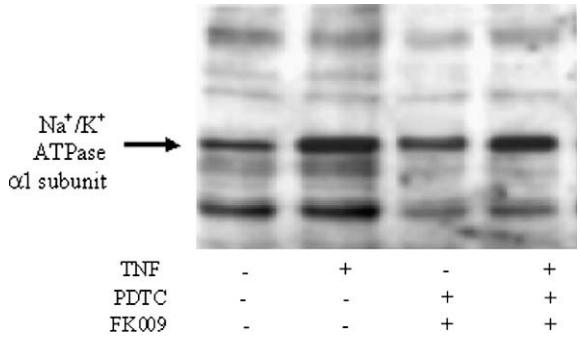

Fig. 5b. Effect of TNF-α in the simultaneous presence and absence of FK009 and PDTC on the activity of the Na^+/K^+ ATPase in HepG2 cells incubated with the cytokine for 4 h. Results are representative of an experiment repeated three times.

full effect of TNF-α on the ATPase activity and expression was maintained (Fig. 5a and b).

Treating the cells with SP 600125 and FK009 concurrently did not lead to any change in the activity or protein expression of the pump (Fig. 6a and b). The up-regulatory effect of TNF-α still appeared in their presence.

Cells treated with the three inhibitors, FK009, SP600125 and PDTC, simultaneously, did not show any change in their Na^+/K^+ ATPase activity or protein expression. However in their presence the effect of TNF was abrogated completely (Fig. 7a and b).

Table 1 summarizes the effect of the different mediators on the pump.

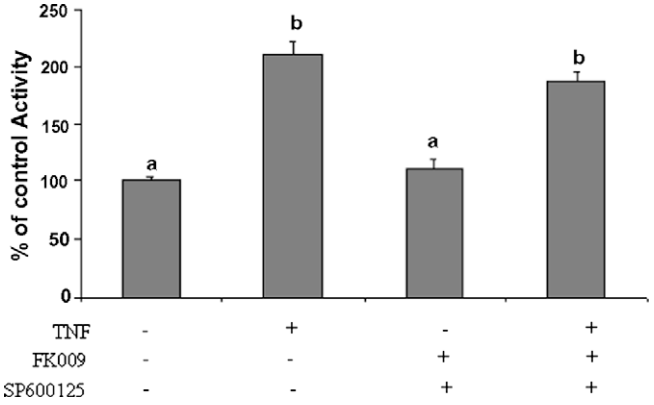

Fig. 6a. Effect of TNF-α in the simultaneous presence and absence of FK009 and SP600125 on the activity of the Na^+/K^+ ATPase in HepG2 cells incubated with the cytokine for 4 h. Values are means ± SE of three observations assayed each in triplicates. Bars not sharing a common letter are significantly different from each other. $P < 0.01$.

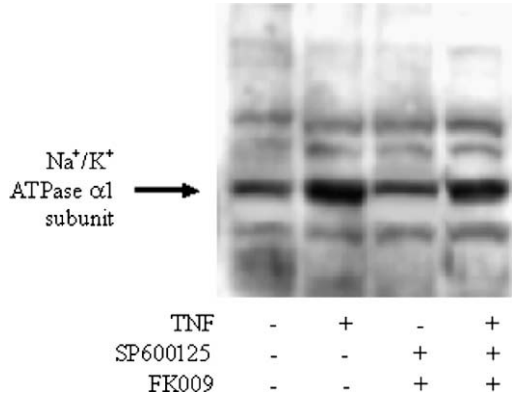

Fig. 6b. Effect of TNF-α in the simultaneous presence and absence of FK009 and SP600125 on the activity of the Na^+/K^+ ATPase in HepG2 cells incubated with the cytokine for 4 h. Results are representative of an experiment repeated three times.

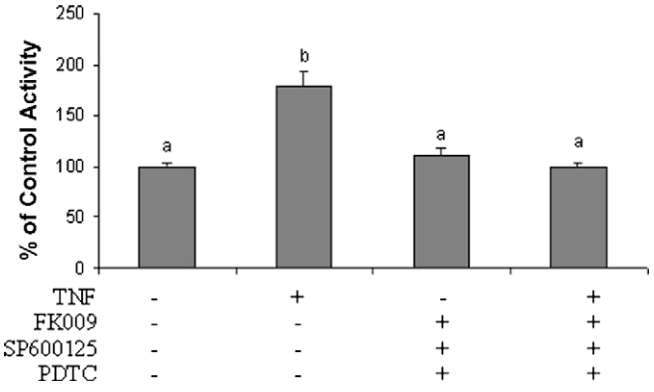

Fig. 7a. Effect of TNF-α in the simultaneous presence and absence of FK009, SP600125 and PDTC on the activity of the Na^+/K^+ ATPase in HepG2 cells incubated with the cytokine for 4 h. Values are means ± SE of three observations assayed each in triplicates. Bars not sharing a common letter are significantly different from each other.

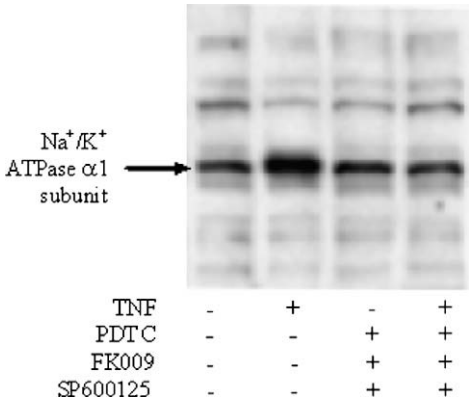

Fig. 7b. Effect of TNF-α in the simultaneous presence and absence of FK009, SP600125 and PDTC on the activity of the Na^+/K^+ ATPase in HepG2 cells incubated with the cytokine for 4 h.

4. Discussion

TNF-α is known to activate different mediators and signaling pathways that culminate in either cell survival or cell death. Activation of the caspase cascade leads to apoptosis [13], while activation of NF-κB, induces genes having anti-apoptotic effects. Many works demonstrated that apoptosis is accompanied by a reduction in the activity of Na^+/K^+ ATPase in various experimental models while stimulation of the ATPase was found to accompany liver regeneration [14]. Although many isoforms of the enzyme are known to exist, the α1 isoform is the one present in hepatocytes [15].

4.1. Time and dose response studies

TNF acted on the Na^+/K^+ ATPase time-dependently (Fig. 1a). Changes in the ATPase activity and protein expression followed a similar trend (Fig. 1a and b), suggesting that changes in activity are due to changes in the number of ATPase molecules. To determine whether these opposite responses are also dose-dependent, cells were treated with different concentrations of TNF-α for 4hrs. At 4 h, TNF-α at doses equal or higher than 40 ng/ml exerted a stimulatory effect on the ATPase (Fig. 1c). Since an increase in pump's activity was reported to occur during liver regeneration [16], it can be speculated that TNF promotes at 4 h cell survival.

The mechanism underlying the early inhibitory response was studied in a previous work [8]. This work is an attempt to unravel the signaling mechanism behind the 4 h-response.

4.2. Signaling mediators

4.2.1. TNF stimulates NF-κB

Inhibition of NF-κB with PDTC abrogated completely the effect of TNF-α. The results imply that the effect of TNF-α on NF-κB is stimulatory because had it been inhibitory, then PDTC would have imitated the effect of TNF-α and an up-regulatory effect would have been observed.

4.2.2. TNF stimulates JNK

Treating the cells with SP600125 (a specific inhibitor of JNK) abrogated only partially the effect of TNF-α (Fig. 3a and b) suggesting the presence of additional mediators that contribute to the effect of TNF on the pump. JNK should be stimulated by the cytokine as was the case with NF-κB and for the same reasons mentioned above for NF-κB.

4.2.3. TNF inhibits caspases

The up-regulatory response to TNF-α still appeared in presence of FK009 (Fig. 4a and b), indicating that either caspases are not involved in TNF action, or if they are, they are inhibited by the cytokine. In the latter case, caspase inhibition should imitate the effect of TNF-α and this is in fact what was observed when FK009 was added alone (Fig. 4a and b). Addition of TNF-α simultaneously with FK009, lead to a similar response to the one observed with each one separately, inferring that caspases are part of the signaling pathway activated by TNF or else an additive effect would have been observed.

It can be concluded that TNF-α acts via stimulation of NF-κB and JNK, and inhibition of caspases. No additional mediators are involved in TNF signaling, since when cells were treated with the cytokine in presence of inhibitors of all three mediators the effect of TNF-α disappeared completely (Fig. 7a and b).

4.3. Positions of the signaling mediators in the pathway

4.3.1. JNK is upstream of NF-κB

It is apparent from the above that caspases, in the basal state, exert an inhibitory effect on the activity and protein expression of the Na^+/K^+ ATPase (Fig. 4a and b).

The effect of JNK is however stimulatory since a down-regulation was observed when it was inhibited with SP 600125. Inhibition of NF-κB did not have any effect on the pump, suggesting that the transcription factor is not involved in its regulation in the basal state.

The fact that the effect of FK009 was abrogated partially with PDTC but fully with PDTC + SP600125, suggests that NF-κB and

Table 1
A summary of the effect of different inhibitors on the Na^+/K^+ ATPase.

TNF-α	PDTC	SP00125	FK009	Effect on ATPase protein expression	Effect on ATPase activity
+				Up-regulation	Up-regulation
	+			No effect	No effect
+	+			No effect	No effect
		+		Down-regulation	Down-regulation
+		+		Reduced up-regulation	Reduced up-regulation
			+	Up-regulation	Up-regulation
+			+	Up-regulation	Up-regulation
	+	+		Down-regulation	Down-regulation
+	+	+		Down-regulation	Down-regulation
	+		+	Reduced up-regulation	Reduced up-regulation
+	+		+	Up-regulation	Up-regulation
		+	+	No effect	No Effect
+		+	+	Up-regulation	Up-regulation
	+	+	+	No effect	No effect
+	+	+	+	No effect	No effect

JNK activate two different pathways that stimulate the pump. It can be postulated that the basal activity of the Na^+/K^+ ATPase is a balance between two stimulatory pathways mediated via JNK and NF-κB, and an inhibitory pathway mediated via caspases. When caspases are inhibited, the stimulatory effect of JNK and NF-κB becomes apparent. When caspases and NF-κB are inhibited concurrently, the up-regulatory effect is reduced because it becomes restricted only to the one caused by JNK. However when JNK and caspases are inhibited concurrently, leaving only NF-κB active, no change is observed in the ATPase activity and expression. As the up-regulatory effect of NF-κB was not observed in this case, it can be postulated that it is activated by either JNK or caspases. Since the effect of FK009 disappeared partially in presence of PDTC but fully in presence of SP600125 and SP600125 + PDTC, it can safely be concluded that JNK is upstream of NF-κB and causes its activation.

When JNK and NF-κB were inhibited simultaneously (Fig. 3a and b), leaving only the caspases active, down-regulation of the pump was observed. This confirms the inhibitory effect of caspases on the ATPase, an effect that was not counterbalanced anymore, in this case, by the stimulatory effect of JNK and NF-κB.

4.3.2. JNK is upstream of caspases

The effect of TNF was abrogated partially in presence of SP600125 alone but reappeared in the simultaneous presence of FK009 and SP600125. It can be speculated that TNF-α inhibits the caspases via activation of JNK, freeing thus the ATPase from their down-regulatory effect. The data indicate also that JNK is upstream of caspases.

4.3.3. NF-κB is upstream of caspases

Inhibition of NF-κB with PDTC abrogated the effect of TNF and although JNK is still active, no up-regulation was observed because it was counteracted by an inhibition caused by active caspases. However, TNF effect reappeared upon simultaneous inhibition of NF-κB and caspases, implying that when NF-κB is active, it inhibits caspases and is upstream of them.

When JNK and NF-κB are inhibited concurrently, the ATPase is down-regulated by TNF-α because the caspases remain active and are not inhibited by NF-κB.

The cytokine could not exert its effect in presence of inhibitors of all three mediators since all stimulatory and inhibitory pathways are blocked. The results infer that no additional mediators are involved in the response to TNF-α.

4.4. Signaling pathway of TNF-α

It can be concluded that TNF-α acts via stimulation of JNK and NF-κB, and inhibition of caspases. NF-κB is upstream of the caspases and inhibits them while JNK is upstream of NF-κB and activates it and is itself activated by TNF-α. TNF activates also NF-κB but this activity is considerably enhanced by JNK. Fig. 8 represents the postulated signaling pathway underlying the effect of TNF-α on the Na^+/K^+ATPase at 4 h.

4.5. Possible implications of the up-regulatory effect of TNF-α on the Na^+/K^+ATPase: roles of the mediators

Up-regulation of the pump by TNF-α is suggestive of an anti-apoptotic state. In fact, in liver regeneration, the activity of the Na^+/K^+ATPase was found to be increased [17,7] and NF-κB activated [7].

The anti-apoptotic effect of NF-κB in hepatocytes has been very well documented and its cytoprotective function has been reported by many studies in various cell types [18–23]. Mice deficient in NF-κB activation have been shown to be more susceptible to hepatocarcinogenesis [24]. By activating NF-κB, the cytokine induces a number of anti-apoptotic genes, including *c-FLIP*, *cIAP1*, *cIAP2*, *A1*, *A20*, *TRAF1*, and *TRAF2* [5].

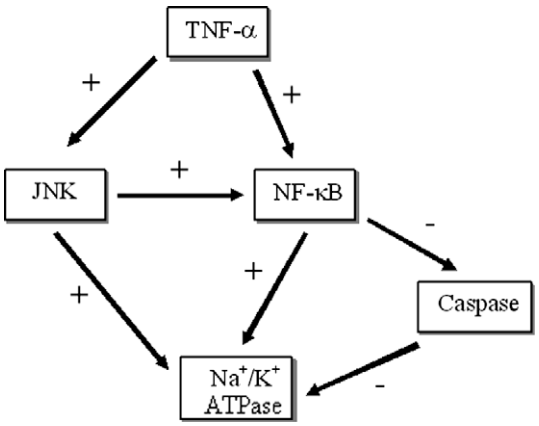

Fig. 8. The signaling pathway underlying the effect of TNF-α on the Na^+/K^+ ATPase in HepG2 cells incubated with the cytokine for 4 h.

On the other hand caspases have been well recognized as key mediators of apoptosis and cell death [25,26]. Since an anti-apoptotic state prevails at 4hrs, the observed inhibition of caspases is expected.

The role of JNK is however till now highly controversial. Many works correlated activation of JNK with cell death. The kinase was shown to play a central role in mediating the TNF-induced apoptotis in hepatocytes [27,28] and developing embryonic forebrain [29,30]. Other studies however, did not find any association between JNK and cell death [31].

In this work JNK activation seems to have an anti-apoptotic role, promoting cell survival and proliferation. These findings are in line with those reporting a role of JNK in oncogenesis [32–34].

The TNF-induced activation of JNK observed in this work is needed to activate in turn NF-κB. One possible mechanism by which the kinase could activate the transcription factor would be by phosphorylating directly or indirectly IκB kinase (IKK) leading to its activation. IKK would in turn phosphorylate the inhibitory protein IκB on serine residues [34] targeting it for ubiquitination and subsequent degradation, and leading consequently to NF-κB activation [18]. Active NF-κB then inhibits caspases. Such an effect has been demonstrated before in fibroblasts by Tang et al. [35].

It can be concluded that TNF-α exerts a time-dependent dual and opposite effect on the pump. The early 1 h response is inhibitory and suggestive of an apoptotic state while the later response at 4 h is stimulatory and suggestive of cell survival conditions. Although in both responses JNK is activated and is the key player, yet it exerts opposite effects on its targets namely NF-κB and caspases which mediate, respectively, cell survival and apoptosis. Caspases are active at 1 h but become inhibited at 4 h. The intriguing question that needs to be addressed is why is there a transient and early activation of caspases that is followed by inhibition? Is this a step in preparation for the anti-apoptotic state that will appear at 4 h? Su et al. [36] demonstrated previously that a deficiency in caspase 8 abolished the activation of NF-κB induced by antigen receptors, Fc receptors, or Toll-like receptor 4 in T, B, and natural killer cells, ascribing thus a role for caspases in the activation of the transcription factor. It would be plausible then to assume that here again, the early and transient activation of caspases aims at triggering the activation of NF-κB which in turn would shut them down.

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