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The NADPH oxidase inhibitor VAS2870 impairs cell growth and enhances TGF-β-induced apoptosis of liver tumor cells

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ABSTRACT

Liver tumor cells show several molecular alterations which favor pro-survival signaling. Among those, we have proposed the NADPH oxidase NOX1 as a prosurvival signal for liver tumor cells. On the one side, we have described that FaO rat hepatoma cells show NOX1-dependent partial resistance to apoptosis induced by Transforming Growth Factor beta (TGF-β). On the other side, we have shown that FaO cells, as well as different human hepatocellular carcinoma (HCC) cell lines, are able to proliferate in the absence of serum through the activation of a NOX1-dependent signaling pathway. The aim of this work was to analyze the effects of NADPH oxidase pharmacological inhibition in liver tumor cells using the inhibitor VAS2870. This compound inhibits dose-dependently autocrine increase of cell number in FaO rat hepatoma cells, and almost completely blocked ROS production and thymidine incorporation when used at 25 μM. Such inhibitory effect on autocrine growth is coincident with lower mRNA levels of EGFR (Epidermal Growth Factor Receptor) and its ligand TGF-α (Transforming Growth Factor-alpha), and decreased phosphorylation of the EGFR itself and other downstream targets, such as SRC or AKT. Moreover, NADPH oxidase pharmacological inhibition also effectively attenuates serum-dependent growth and phosphorylation of AKT and ERK. Importantly, these inhibitory effects on either autocrine or serum-dependent cell growth are observed in several human HCC cell lines. Finally, we have observed that VAS2870 is also effective in enhancing apoptosis induced by a physiological stimulus, such as TGF-β. In summary, NADPH oxidase pharmacological inhibition could be considered a promising tool in the treatment of liver cancer.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the cancers with highest mortality worldwide. Even if the pathogenesis of this disease is highly complex, it is generally well accepted that, in all cases, it exists an unbalance between proliferation and cell death, mainly caused by overactivation of survival pathways [1]. In fact, the main reported signaling pathways implicated in HCC pathogenesis are related to proliferation and angiogenesis [2]. Even though risk factors associated with HCC are well documented, prognosis is usually poor mainly due to the lack of effective therapies. For these reasons, the discovery of new treatments to expand the present therapeutic options is essential in the future handling of the disease.

The NOX family of NADPH oxidases includes 7 different enzymes whose main function is the production of Reactive Oxygen Species (ROS). These enzymes are widely expressed in numerous tissues and play different roles including cell signaling, gene expression regulation, cell death, differentiation and growth [3]. Most of NOX functions are related to signal transduction from membrane receptors and, consequently, they are activated in response to extracellular signals, such as cytokines or growth factors [4].

Hepatocytes express different members of the NOX family. In fact, we have previously described that fetal rat hepatocytes show NOX1, NOX2, and NOX4 expression in basal conditions [5] showing apparent opposite roles in the control of liver cell death. Thus, NOX4 is necessary for the triggering of apoptosis induced by a physiological stimulus, such as the Transforming Growth Factor-beta (TGF-β) [6] or antineoplastic drugs, such as doxorubicin [7]. On the contrary, NOX1 might be involved in protecting cells from TGF-β proapoptotic signals in both fetal hepatocytes and hepatoma cells [5,8]. Importantly, we have recently reported an essential role for NOX1 in controlling autocrine growth through the

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Epidermal Growth Factor Receptor (EGFR), a mechanism which seems to be specific for liver tumor cells as compared to non tumoral hepatocytes [9].

According to this, the aim of the present work was to investigate whether NADPH oxidase pharmacological inhibition shows antitumor efficacy in vitro, evaluating both its antiproliferative and proapoptotic activities in rat and human liver cancer cells. Results will show relevance in the design of new therapeutic strategies for liver cancer.

2. Materials and methods

2.1. Cell culture conditions

FaO rat hepatoma cells, Hep3B and HepG2 human hepatocarcinoma cells, SK-HEP-1 human liver adenocarcinoma, and PLC/PRF/5 human liver hepatoma were obtained from the European Collection of Cell Cultures (ECACC). For cell culture, the following media were used: F12 Coon's modified medium (Sigma, Madrid Spain) for FaO, MEM (Sigma, Madrid Spain) for Hep3B and HepG2, 1 mM pyruvate-supplemented MEM for SK-HEP-1 and DMEM (Lonza, Basel, Switzerland) for PLC/PRF/5. Cell lines were grown in medium supplemented with 10% fetal bovine serum and maintained in a humidified atmosphere of 37 °C, 5% CO_2. To test autocrine growth, cells were serum deprived at 40% confluence and, when indicated, the NADPH oxidase inhibitors Apocynin (300 μM; Sigma, Madrid, Spain) or VAS2870 (provided by Vasopharm BIOTECH GmbH, Wurzbürg, Germany) were added 30 min before serum deprivation and maintained along the experiment. For TGF-β experiments, cells at 70% confluence were serum deprived for 16 h and treated with 2 ng/ml TGF-β (Calbiochem, La Jolla, CA, USA) in the presence or absence of the EGFR inhibitor AG1478 (20 μM; Calbiochem) or VAS2870 (25 μM), which were added 30 min prior to TGF-β.

2.2. Analysis of cell number

Cell number was analyzed after crystal violet staining (0.2% in 2% ethanol), as previously described [10].

2.3. Proliferation measurement by [^{3}H]-thymidine incorporation

Cells were treated during 48 h as indicated in the presence of 1 μCi/ml, 1 μM thymidine (Hartmann Analytic GmbH, Braunschweig, Germany). Then, cells were incubated for 20 min at 4 °C with 10% trichloroacetic acid, washed twice with 70% ethanol and let them dry for at least 1 h. Finally, the acid precipitated material is resuspended in a buffer containing 2% Na_2CO_3, 0.1 N NaOH, 0.5% SDS and radioactivity was measured in a scintillation counter 1209 Rackbeta (Wallac, Turku, Finland) diluting 100 μL of acid precipitated material in 5 ml of scintillation liquid.

2.4. Measurement of intracellular redox state

The oxidation-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen, Carlsbad, CA, USA) was used to analyze the total intracellular content of ROS as previously described [8]. Fluorescence was measured in a Microplate Fluorescence Reader Fluostar Optima and expressed as percentage of control after correction with protein content.

2.5. Analysis of caspase-3 activity

Caspase-3 activity was analyzed fluorimetrically upon incubation of 20 μg of cell lysates with 6.6 μg/mL Ac-DEVD-AMC (BD Pharmingen, Franklin Lakes, NJ, USA) for 2 h at 37 °C as described previously [8]. Protein concentration of cell lysates was determined using the Bio-Rad protein assay kit (Hercules, CA, USA). Results are calculated as units of caspase-3 activity per microgram of protein per hour and expressed as fold induction relative to control.

2.6. Analysis of gene expression

RNeasy mini kit (Qiagen, Valencia, CA, USA) was used for total RNA isolation. Reverse transcription (RT) was carried out using the High Capacity Reverse Transcriptase kit (Applied Biosystems, Foster City, CA, USA), with 500 ng of total RNA from each sample for complementary DNA synthesis. Semiquantitative PCRs were performed using specific primers for rat samples:

NOX1: F 5′TTACTACTGCCTCCATCAAGC3′; R 5′GCTGCATACATCACTGTCACG3′
NOX2: F 5′TCAAGTGTCCCCAGGTATCC3′, R 5′TTCACTGGCTGTACCAAAGG3′
NOX4: F 5′TTACTACTGCCTCCATCAAGC3′, R 5′GGAATGATTGGATGTCTCTGC3′
Cyclin D1: F 5′ATGTTCGTCCGGTCTAAGATG3′, R 5′TGCGGATGATCTGCTTGTTC3′
EGFR: F 5′AAACTCTTCGGGACGCCC AATC3′, R 5′TGGCGATGGATGGGATCTTTG3′
HBEGF: F 5′CGGTGGTGC TGAAGCTCTTTC3′, R 5′TGGTAACC AGGGAGGCAGTG3′
TGF-α: F 5′TGGTGCAGGAAGAGAAGC3′, R 5′TGACAGCAGTGGATCAGC3′
Albumin: F 5′CTGCCGATCTGCC CTCAATAG3′, R5′TGCCCACTCTTCCCAGGTTTCT3′

PCR products were obtained after 30–35 cycles of amplification at annealing temperatures of 57–62 °C, and analyzed by 1.5% agarose gel electrophoresis. Expression of albumin was analyzed as a loading control, as indicated. The –RT channel contained RNA that had not been treated with the RT mixture and is shown as a specificity control.

For Real-Time quantitative PCR, expression levels were determined in duplicate in an ABIPrism7700 System following manufacturer's protocol. Pre-designed Taqman® primers for NOX1 (Rn00586652_m1), EGFR (Rn00580398_m1), TGF-α (Rn00446234_m1) and housekeeping GAPDH (Rn99999916_s1) and Taqman® Universal Master Mix were used. All real time reagents were from Applied Biosystems (Foster City, CA, USA).

2.7. Western blot analysis

Total protein extracts and Western blot procedure were carried out as previously described [11]. The antibodies used were: mouse anti-β-actin (clone AC-15); rabbit anti-phospho-Akt (Ser473), rabbit anti-Akt, rabbit anti-phospho-EGFR (Tyr1068), rabbit anti-EGFR, rabbit anti-phospho-p44/42 MAPK (Thr202/Tyr 204), anti-p44/42 MAP Kinase, rabbit anti-phospho-SRC Family (Tyr416). All antibodies were from Cell Signaling Technology (Beverly, MA, USA), except anti-β-actin from Sigma (Madrid, Spain). Antibodies were used at 1:1000, except β-actin (1:3000). Protein concentration was measured with BCA™ Protein Assay kit (Pierce, Rockford, USA).

3. Results

3.1. The NADPH oxidase inhibitor VAS2870 blocks autocrine cell growth of FaO rat hepatoma cells

As we had previously described, FaO rat hepatoma cells showed autocrine proliferation in absence of serum, doubling the initial cell number in about 48 h upon serum withdrawal (Fig. 1A).

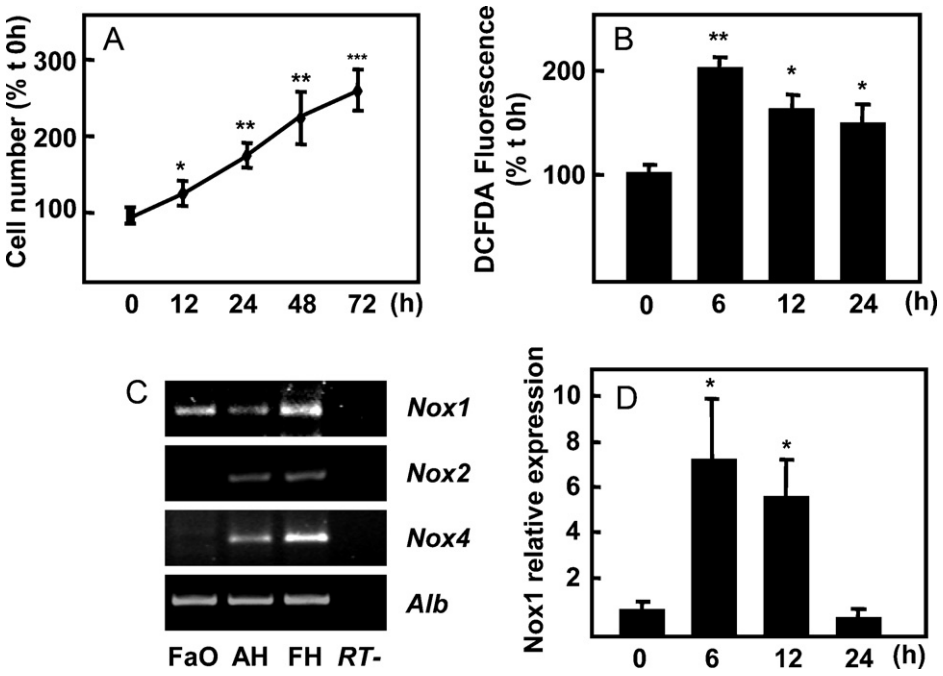

Fig. 1. FaO rat hepatoma cells show autocrine proliferation correlating to ROS production and NOX1 up-regulation. (A), (B), (D) FaO rat hepatoma cells were serum-depleted for the indicated times and it was measured: cell number (A), ROS production (B) and NOX1 expression by real time PCR (D). Data were calculated relative to zero time and represent the mean ± SEM of three independent experiments. Student's *t*-test calculated versus zero time: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. In (C), RT-PCR comparing NADPH oxidases expression of FaO cells, adult rat hepatocytes (AH) and fetal hepatocytes (FH).

Interestingly, cell growth was accompanied by ROS production, measured by DCFDA oxidation, with a maximal peak of ROS production detected at 6 h (Fig. 1B). Looking for the source of ROS, we have observed that FaO rat hepatoma cells only expressed the NADPH oxidase NOX1, differently to adult or fetal rat hepatocytes, which also presented NOX2 and NOX4 expression (Fig. 1C). Consequently, we have found that ROS production is coincident with a NOX1 up-regulation with similar timing (Fig. 1D). Since we have previously demonstrated that NOX1 silencing by a siRNA approach could inhibit autocrine growth [9], we wanted to test the

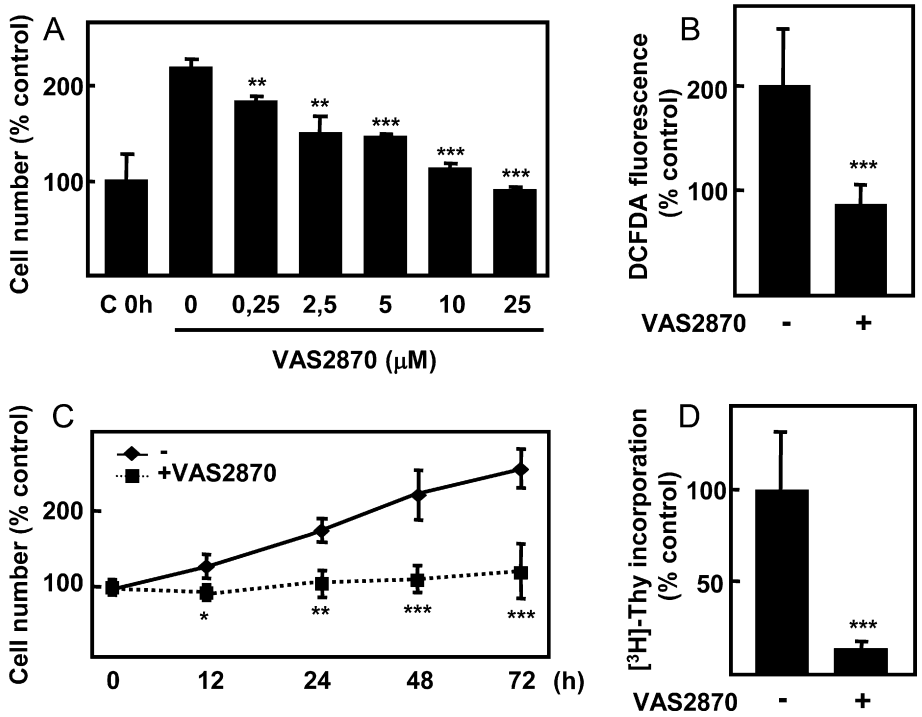

Fig. 2. The NADPH oxidase inhibitor VAS2870 blocks autocrine cell growth and ROS production. (A) FaO cells were serum depleted and treated with the indicated concentrations of the NADPH oxidase inhibitor VAS2870. After 48 h, cell number was measured. The concentration of 25 μM was selected for the rest of experiments. (B) ROS production at 12 h. (C) Cell number at the indicated times. (D) DNA synthesis at 48 h. Data were calculated relative to zero time, except in (D) were it was calculated respect to untreated cells, and represent the mean ± SEM of three independent experiments. Student's *t*-test calculated versus zero time or untreated cells in (D): $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

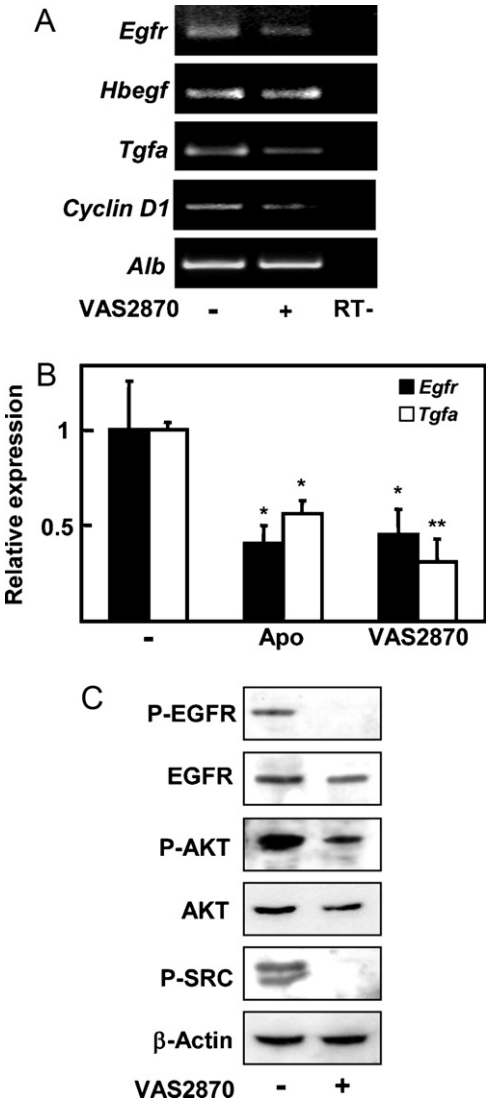

Fig. 3. Pharmacological inhibition of NOX1 with VAS2870 down-regulates the EGFR pathway. FaO cells were treated for 12 h with the NADPH oxidase inhibitors VAS2870 or Apocynin (300 μM, in (B)) prior to serum depletion. (A) RT-PCR. (B) EGFR and TGF-α expression measured by Real-time PCR. Data represent the mean ± SEM of four independent experiments. Student's *t*-test calculated as pretreated versus non-pretreated cells: $^{*}p < 0.05$; $^{**}p < 0.01$. (C) Western blot of total lysates. Albumin (Alb) and β-actin were used as loading control.

efficiency of the NADPH oxidases pharmacological inhibition in the same experimental conditions. For this purpose, we decided to use the Vasopharm BIOTECH GmbH drug VAS2870, a pharmacological NOX inhibitor, which has been useful to inhibit NADPH oxidases in preclinical assays both in vitro and *in vivo* [12,13]. This compound showed a dose-dependent inhibitory effect on cell growth in FaO cells (Fig. 2A), which was maximal at the 25 μM dose. In fact, this concentration was able to completely block ROS production (Fig. 2B) and also cell growth measured both by cell number change kinetically and DNA synthesis by [^{3}H]-thymidine incorporation (Fig. 2C and D). Importantly, the high degree of growth inhibition correlated to much lower expression of cyclin D1, EGFR and TGF-α, analyzed at the mRNA level (Fig. 3A). Moreover, Apocynin, a widely used NOX inhibitor, produced a similar decrease in the EGFR and TGF-α mRNA levels when compared to VAS2870 (Fig. 3B). Interestingly, phosphorylation of the EGFR itself and some downstream targets such as c-SRC and AKT was almost completely blocked in the presence of VAS2870 (Fig. 3C). All these results suggest that NADPH oxidase pharmacological inhibition effectively reduce FaO cells autocrine growth through inhibiting the EGFR pathway at the EGFR and TGF-α expression level.

3.2. The NADPH oxidase inhibitor VAS2870 blocks serum-dependent cell growth of FaO rat hepatoma cells

Since NOX enzymes have been described to modulate not only autocrine cell growth but also proliferation in response to serum and/or mitogenic signals, we decided to test whether VAS2870 was equally effective in inhibiting serum-dependent cell growth at the same concentration used for autocrine growth inhibition tests in FaO cells. As shown in Fig 4, the addition of 25 μM VAS2870 inhibited cell number change (Fig. 4A) and DNA synthesis (Fig. 4B), although growth inhibition in these conditions was less remarkable than that observed upon serum withdrawal. Importantly, basal ROS amount was slightly decreased when VAS2870 was present (Fig. 4C). Those events correlated to decreased AKT and ERK1/2 phosphorylation (Fig. 4D), kinases widely linked to proliferation signaling. However, EGFR phosphorylation was not affected by the inhibitor in the presence of serum. In summary, VAS2870 inhibits both autocrine and mitogen-dependent proliferation probably through NOX1 inhibition, although the downstream molecular mechanisms mediating both processes might be different.

3.3. VAS2870 inhibits proliferation of different human hepatocellular carcinoma (HCC) cell lines

To better evaluate the possible applicability of NOX pharmacological inhibition for hepatocellular carcinoma treatment, we decided to test VAS2870 effects in different human HCC cell lines. We observed that, similarly to FaO rat hepatoma cells, the four tested cell lines presented autocrine proliferation in absence of serum, measured both as cell number change and DNA synthesis, which was greatly inhibited in the presence of VAS2870 (Fig. 5A). Importantly, no apoptosis was detected, measured as caspase-3 activity (results not shown). Moreover, as observed in FaO cells, VAS2870 inhibitory effect was also detected in the presence of serum (Fig. 5B). In summary, VAS2870 efficiently blocked both autocrine and serum-dependent growth of human liver cancer cells.

3.4. VAS2870 pretreatment enhances TGF-β-mediated apoptosis of FaO rat hepatoma cells

By means of siRNA technology, we had previously reported that FaO rat hepatoma cells show partial resistance to TGF-β-induced apoptosis due to NOX1-dependent ROS [8], process dependent on EGFR activation. For this reason, we wanted to know whether we could achieve a restoration of the apoptotic response to TGF-β when cells were pretreated with VAS2870. As shown in Fig. 6, FaO cells partially responded to TGF-β-induced apoptosis, detected by decreased cell number and caspase-3 activation, response which is enhanced when cells were pretreated with the EGFR inhibitor AG1478 as we have previously described [8]. In a similar manner, NADPH oxidase inhibition with VAS2870 also potentiated TGF-β-induced apoptosis with comparable efficacy, measuring both the cell number and caspase-3 activity.

In summary, NADPH oxidase pharmacological inhibition effectively decreases proliferation of rat hepatoma and human hepatocellular carcinoma cells, both autocrine and serum-dependent growth, and also enhances the response of hepatoma cells to a physiological proapoptotic stimulus, namely TGF-β.

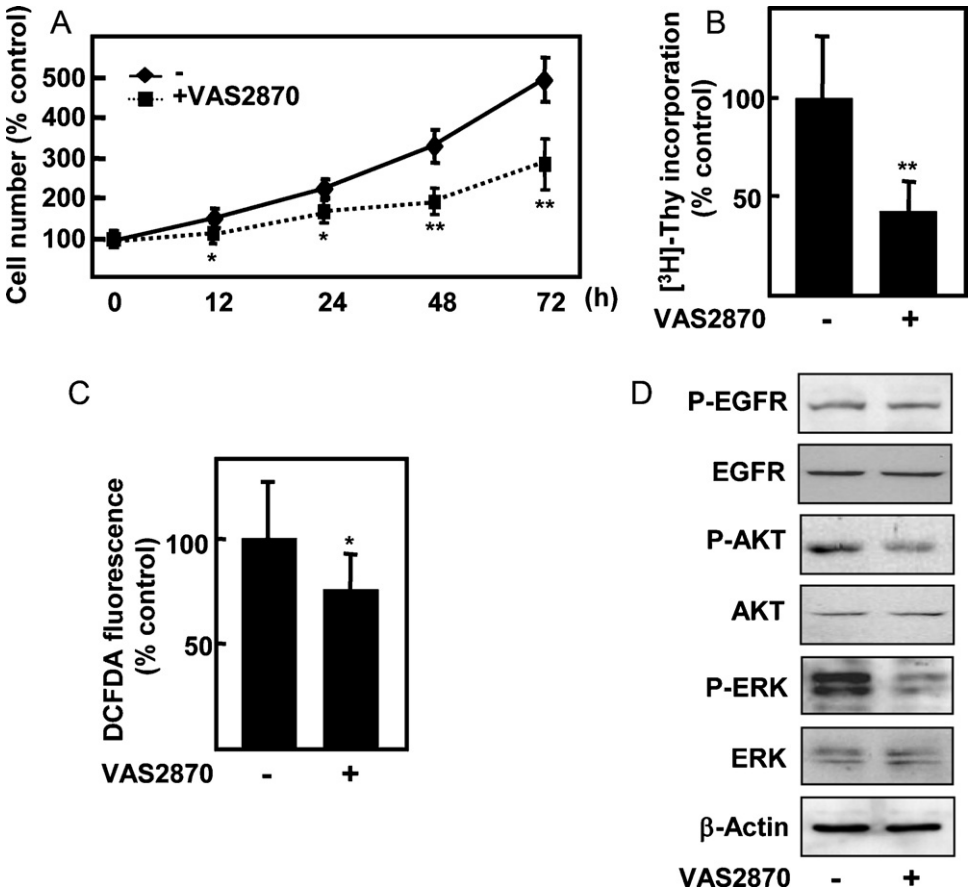

Fig. 4. The NADPH oxidase inhibitor VAS2870 decreases serum-induced cell growth and AKT/ERK phosphorylation. FaO cells were treated with 25 μM VAS2870 in the presence of serum, and it was determined. (A) Cell number at the indicated times. (B) DNA synthesis at 48 h. (C) ROS production at 12 h. (D) Phosphorylation of EGFR, AKT and ERK1/2 by Western blot at 12 h. β-Actin was used as loading control. Data were calculated relative to untreated cells, except in (A) were it was calculated respect zero time, and represent the mean ± SEM of three independent experiments. Student's *t*-test calculated versus untreated cells or zero time in (A): $^{*}p < 0.05$; $^{**}p < 0.01$.

4. Discussion

Hepatocellular carcinoma is a major cause of cancer-related deaths worldwide. Risk factors associated with HCC are well documented, but usually prognosis is negative due to high malignancy of the lesion once diagnosed, and lack of effective therapies [14]. All these facts mean that only 30–40% of patients are suitable for curative treatments, generally corresponding to liver resection or transplantation [15]. For these reasons, the discovery of new treatments to expand the present therapeutic options is essential in the future handling of the disease.

The main reported signaling pathways implicated in HCC pathogenesis are EGFR/RAS, C-Met, IGF-1, AKT/mTOR, VEGF y PDGFR, all of them related to proliferation and angiogenesis [16]. Interestingly, endogenous ROS production has been described to be very relevant in the cell growth of hepatic tumors [17,18]. Moreover, animal models of hepatocarcinogenesis induction mostly have ROS production in common, despite of the cellular targets or cytotoxic effects [19]. In fact, tumor growth inhibition has been described to occur upon antioxidants dietary supplementation in animal models [20]. Moreover, a NADPH oxidase system-derived ROS production has been proposed as main mediator in hepatocarcinogenic processes occurring in different animal models, such as the TGF-alpha/c-myc transgenic mice [20], or exposure to carcinogens such as diethylnitrosamine [21].

NADPH oxidases are enzymes catalyzing the generation of superoxide or hydrogen peroxide using NADPH as electron donor. They were cloned by homology to the phagocytic NADPH oxidase *gp91phox* (NOX2) and have been described to function as signaling molecules in non-phagocytic cells [22]. In the liver, the isoforms NOX1, NOX2 and NOX4 are expressed by fetal and adult hepatocytes ([5], Fig. 1C), although they might have opposite effects controlling cell death: NOX4 favoring apoptosis whilst NOX1 favors survival [6,8]. Interestingly, tumoral hepatocytes show an altered NOX expression pattern, indicating that they have developed mechanisms to repress the pro-apoptotic NOX4 at the transcriptional level and expressing NOX1 or other isoforms [8,23]. In this sense, we had postulated a pro-tumorigenic role for NOX1 based on the next evidence: (1) NOX1 protects hepatoma cells from physiological proapoptotic signals such as TGF-β [8]; and (2) NOX1 might be controlling autocrine growth through the EGFR, a mechanism which seems to be specific for liver tumor cells as compared to non tumoral hepatocytes [9].

For these reasons, we wanted to test in vitro the potential antitumor efficiency of VAS2870, the only validated low-molecular weight pharmacological NOX inhibitor [13,24]. First of all, we wanted to measure VAS2870 efficiency in inhibiting autocrine growth of FaO rat hepatoma cells. We corroborated the ROS production concomitant to NOX1 induction during the first hours of autocrine growth (Fig. 1), which was almost totally blocked when VAS2870 was present (Fig. 2). Importantly, EGFR and TGF-α expression levels, and also phosphorylation of EGFR, AKT and SRC were diminished with high efficiency (Fig. 3). Indeed, effects of pharmacological inhibition were equivalent to those obtained by siRNA technology, but more pronounced. High efficacy of this drug might be the result of a full and sustained inhibition of cellular NADPH oxidases, compared to partial and transient ROS inhibition obtained with knock-down experiments.

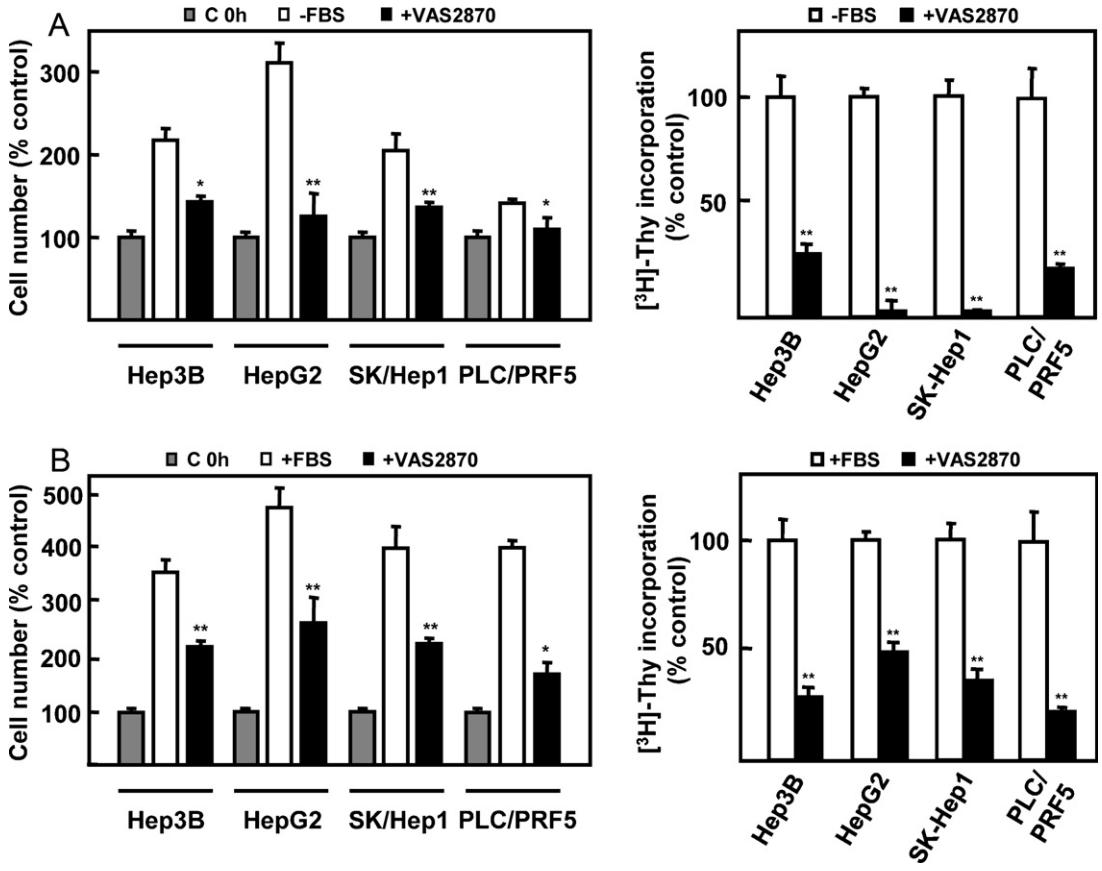

Fig. 5. Treatment with VAS2870 inhibits both autocrine and serum-mediated cell growth of different human hepatocellular carcinoma (HCC) cell lines. Hep3B, HepG2, SK-Hep1 and PLC/PRF5 cells were treated with 25 μm VAS2870 in absence (A) or presence (B) of serum, and cell growth was determined after 48 h by cell number determination (left panels) or DNA synthesis (right panels). Data were calculated relative to zero time for cell number and respect to untreated cells for DNA synthesis, and represented as the mean ± SEM of three independent experiments. Student's *t*-test calculated versus control cells: $^{*}p < 0.05$; $^{**}p < 0.01$.

NOX proteins have been described to transmit signals downstream receptor tyrosine kinases (RTK), such as PDGFR, VEGFR, EGFR, bFGFR and insulin [4], all of them related to the pathogenesis of HCC, as stated above. Those pathways have considerable crosstalks and are redundant in downstream molecules, reason why the only effective chemotherapeutic agent for treatment of patients with non-resectable tumors is the multikinase inhibitor sorafenib [25]. Thus, considering that different RTK might signal through NADPH oxidases, it is reasonable to think that NOX inhibition could affect the downstream signaling through one or several of those receptors at the same time. The results of growth inhibition obtained in the presence of serum (Figs. 4 and 5B) also support such hypothesis: (1) on the one hand, serum contains multiple mitogenic molecules and growth factors, so we could consider that more than one pathway is being activated in such conditions; (2) as shown in Fig. 4D, VAS2870 inhibits both AKT and ERK phosphorylation, previously described as targets of NOX-dependent proliferation in the presence of serum [26], without affecting the phosphorylation of the EGFR itself. This fact might also suggest that NADPH oxidases are acting downstream tyrosine kinase receptors. It is worthy to note that VAS2870 inhibitory effect on NOX enzymes is not isoform-specific [13] and the final result might be a combinative effect of inhibiting all the isoforms expressed in the cell. The response to VAS2870 in the human HCC cell lines included in our study, whose NADPH oxidase expression pattern is more complex than the one detected for FaO cells, indicate the relevance of the proliferative role of NOXes in human liver tumor cells. Indeed, a role for NOX3 in the proliferative response to insulin has been reported in HepG2 cells [27].

Importantly, results obtained with the NADPH oxidase inhibitor VAS2870 in human HCC cell lines (Fig. 5) show the high efficacy of this compound regardless the mutational status of p53 or RAS/RAF pathway. On the one hand, we have chosen for our experiments HepG2 and SK-Hep1 cells presenting over-activation of RAS and B-RAF, respectively [28,29], and we found a high inhibition of the proliferation rate upon incubation with VAS2870 both in the presence or absence of serum. On the other hand, Hep3B and PLC/PRF5 cells, p53 defective, also show high inhibition of growth. Importantly, the use of VAS2870 has no toxicity when applied to non tumoral hepatocytes (not shown).

Finally, we have also shown how VAS2870 treatment enhances the apoptotic response induced by TGF-β in FaO cells (Fig. 6). In normal conditions, this cytokine is considered to act as a liver tumor suppressor, but many tumor cells acquire resistance to its proapoptotic effects, favoring response to this cytokine in terms of malignancy [30]. In addition to survival signals via NOX1 in hepatoma cells [8], TGF-β is able to induce migration of different tumor cells via NOX proteins [31,32] and also promote oxidative damage upon hepatitis C virus, frequently associated with later HCC [33,34]. Thus, inhibiting NADPH oxidases might prevent those events and promote beneficial effects in cases of liver cancer.

In summary, results presented in this manuscript indicate that NADPH oxidase pharmacological inhibition with VAS2870, effectively impairs both autocrine and mitogen-dependent growth of liver tumor cells, attenuating downstream intracellular signals

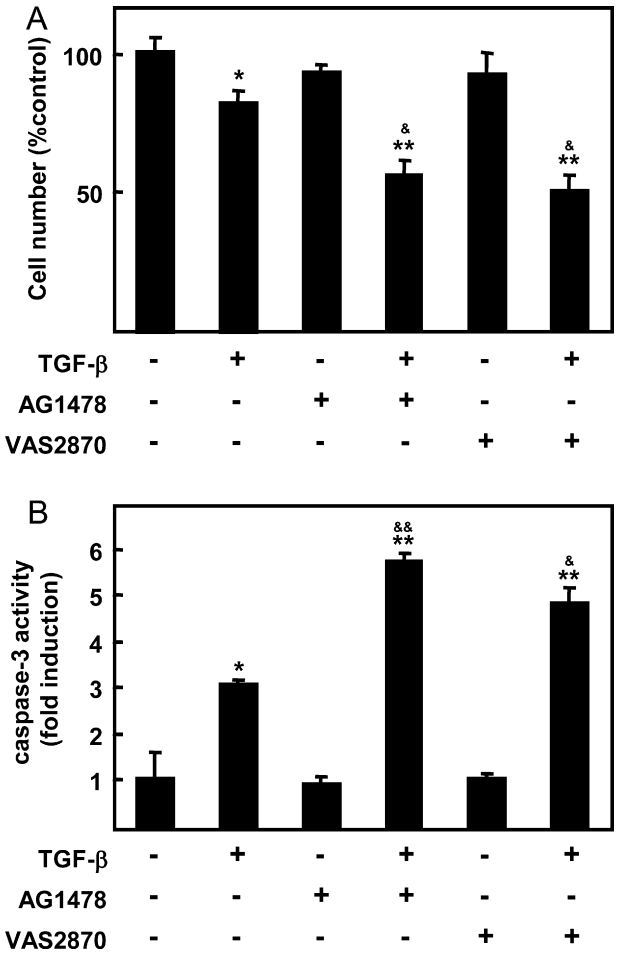

Fig. 6. VAS2870 enhances TGF-β-induced apoptosis in FaO cells, to the same extent as EGFR inhibition with AG1478. FaO cells were serum-depleted for 12 h, pretreated for 30 min with either 25 μM VAS2870 or 20 μM AG1478, and then incubated in the presence or absence of 2 ng/ml of TGF-β. (A) Caspase activity at 16 h. (B) Cell number at 24 h. Data were calculated relative to untreated cells and represent the mean ± SEM of three independent experiments. Student's *t*-test calculated versus control cells ($^{*}p < 0.05$) or TGF-β-treated cells ($^{\&}p < 0.05$; $^{\&\&}p < 0.01$).

that contribute to cell proliferation. Moreover, VAS2870 treatment also enhances apoptosis induced by TGF-β, a physiological proapoptotic stimulus. Indeed, NADPH oxidase pharmacological inhibition might be a promising therapeutic approach for hepatocellular carcinoma.

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