Cytostatic response of HepG2 to 0.57 MHz electric currents mediated by changes in cell cycle control proteins

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Abstract. The capacitive-resistive electric transfer (CRet) therapy is a non-invasive technique that applies electrical currents of 0.4-0.6 MHz to the treatment of musculoskeletal injuries. Although this therapy has proved effective in clinical studies, its interaction mechanisms at the cellular level still are insufficiently investigated. Results from previous studies have shown that the application of CRet currents at subthermal doses causes alterations in cell cycle progression and decreased proliferation in hepatocarcinoma (HepG2) and neuroblastoma (NB69) human cell lines. The aim of the present study was to investigate the antiproliferative response of HepG2 to CRet currents. The results showed that 24-h intermittent treatment with 50 μA/mm^2 current density induced in HepG2 statistically significant changes in expression and activation of cell cycle control proteins p27^{Kip1} and cyclins D1, A and B1. The chronology of these changes is coherent with that of the alterations reported in the cell cycle of HepG2 when exposed to the same electric treatment. We propose that the anti-proliferative effect exerted by the electric stimulus would be primarily mediated by changes in the expression and activation of proteins intervening in cell cycle regulation, which are among the targets of emerging chemical therapies. The capability to arrest the cell cycle through electrically-induced changes in cell cycle control proteins might open new possibilities in the field of oncology.

Introduction

Recently, research on capacitive electrical therapies for cancer treatment has experienced significant growth. Capacitive therapies, also known as capacitive diathermy, apply radio-frequency currents at 6.78, 8 or 13.56 MHz for thermal treatment of a number of ailments. With that purpose, the currents are transmitted through adhesive or inserted, intra- or extra-corporeal capacitive plates, to warm up the patient's internal tissues. Clinical studies with these capacitive therapies have shown increased survival of patients undergoing combined treatments with electric therapy and chemotherapy or radiotherapy, when compared to control patients treated with chemotherapy or radiotherapy alone (1-7).

The capacitive-resistive electric transfer therapy CRet is similar to capacitive therapies but, unlike them, CRet induces hyperthermia in the target tissues through the use of mobile electrodes for transdermal application of electric currents within the 0.4-0.6 MHz frequency range. This therapy has proved effective in restoring damaged tissues including muscle, tendon or vessels (8). Additionally, preliminary tests conducted in cancer patients suffering from glioblastoma multiforme have shown that CRet treatment induced necrosis and decreased tumor growth, which was interpreted as indicative of an oncostatic ability of the therapy (9,10). Despite these indications of potential oncological applications of CRet, the current knowledge about the cellular response to this and other radiofrequency electrothermal therapies is still very poor. In fact, only a few, minor *in vitro* effects have been described for fields at frequencies ≥0.1 MHz (11-13). Consequently, it has been generally assumed that such fields have no relevant biological effects. Nevertheless, recent studies have reported that low intensity electric fields within the 0.1-0.3 MHz frequency range may significantly inhibit cell growth in different human and rodent cancer lines, as well as in solid tumors of human patients (14-16).

In previous studies by our group the cellular response to 24-h, intermittent stimulation with 0.57 MHz electric currents at a subthermal dose of 50 μA/mm^2 was investigated. The *in vitro* experimental protocol was designed by scaling the exposure intervals to simulate the treatment cycle applied in CRet therapies (Fig. 1). The 50 μA/mm^2 current density used in these *in vitro* experiments is within the range of thermal treatments applied to patients. However, in our experimental conditions such doses do not cause temperature increase of the cells due to the configuration of the exposure setup and to the electric properties to the culture medium (8). This represents a significant methodological advantage, allowing us to study the cellular response to the electric stimulus alone, without increasing the temperature of the medium, which could act as a confounding factor. We have reported that such treatment can induce statistically significant responses,

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at the cellular and molecular levels, in neuroblastoma (NB69) and hepatocarcinoma (HepG2) cell lines from human origin, when exposed during the exponential growth phase (8,17). In NB69 cells the electric stimulus caused increased rate of necrosis followed by decreased cell proliferation, this response being attributable to alterations in cell cycle progression (17).

In HepG2, stimulation with the same electrical parameters and using the same, above described protocol, induced decreased cell number without changing significantly the cell death rate. Thus, it is conceivable that the electrically induced cytostatic response is mediated by a transient arrest or slowdown of the cell cycle, at least in a sensitive fraction of the cellular population. The effect on the cell cycle occurs in early stages of exposure and is detectable by flow cytometry as an increase in the rate of cells in S phase during the first 12 h of treatment (Fig. 2). Provided that: i) during these first 12 h, the cellular population in late stages of the cycle (G2 and M) decreases in the treated samples when compared to that in controls, and ii) the proliferation rate of the culture is reduced in the treated samples, the above described accumulation of cells in S phase, of DNA synthesis, is interpreted as a slowing of cell cycle in the affected population. After the 24-h treatment a significant decrease in the population of cells in S phase was observed, together with a slight, though statistically significant increase in the proportion of cells in G0/G1. This could be interpreted as a slowdown or arrest of the cell cycle potentially mediated by alterations in cycle control proteins (8).

The present work investigates the expression and activation, during and after CRet treatment, of three key proteins in cell cycle regulation: cyclin D1, which regulates the G1 phase and its transition to S, and is synthesized in response to exogenous factors (18-22), cyclin A, which controls the S and G2 phases (23,24) and cyclin B1, involved in the regulation of the initiation and control of early mitotic stages (25). Since these cyclins become activated by binding to cyclin-dependent kinases or CDKs, the present study also analyzes the expression and activation of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor (CKI). This protein regulates the activity of cyclin-CDK complexes during G1 and S phases, induces G1 phase arrest and, in proliferating cells, can become overexpressed and activated under certain conditions of damage or stress (26). If the electric stimulus induced changes in expression and/or activation of these cell cycle control proteins, it is likely that such an effect was the cause of the reported alterations in cell cycle progression of HepG2 and of the subsequent decline in cell proliferation. This hypothesis is strongly supported by herein reported results, which reveal significant sequential changes in the cellular expression and activation of cyclins D1, A and B1, and of inhibitor $p27^{Kip1}$ during and after stimulation with weak, 0.57 MHz electric currents.

Materials and methods

Exposure system and experimental design. The experimental protocol and exposure system have been described in a previous report (8). Briefly, HepG2 cells (ECACC, Salisbury, UK) were maintained in flasks, in standard culture conditions. In all experimental runs, samples were seeded at a 10^5 cells/ml density in 60 mm ø Petri dishes and incubated in a 5% CO_2, 37˚C and 100% RH atmosphere. The exposure to CRet electric currents was carried out through pairs of sterile, stainless steel electrodes, designed ad hoc for *in vitro* stimulation (Fig. 3). At day four after seeding, when the cultures had reached the exponential growth phase, the electrodes were inserted both in experimental and control dishes. The experimental dishes were connected in series to an INDIBA stimulator model MD500 (INDIBA S.A., Barcelona, Spain). During 12- or 24-h intervals, the experimental samples were treated every 4 h with a 5-min pulse of 0.57 MHz, sine wave electric current, under controlled conditions of temperature, humidity and CO_2. This 5 min on/4 h off exposure cycle corresponds to a 1:12 scale of the 60-min treatment administered every second day, currently applied to patients undergoing CRet therapy. Considering the geometry of the Petri dish, the conductivity of the media and the meniscus effect, the average

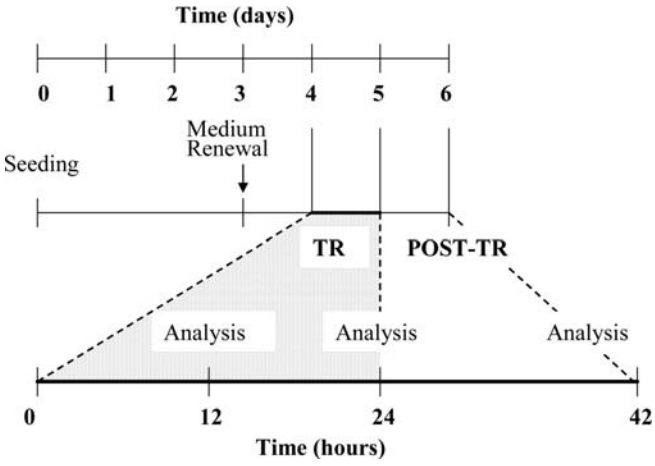

Figure 1. Schematic representation of the experimental protocol. Cells were seeded on day 0. On day 4 the samples were either sham-exposed or treated (TR) simultaneously for 12 or 24 h with 5-min pulses of 0.57 MHz, sine wave current at a density of 50 μA/mm^2. The treatment or sham-exposure interval was followed by an 18-h lapse of additional incubation (42 h) in the absence of electric stimulus (POST-TR).

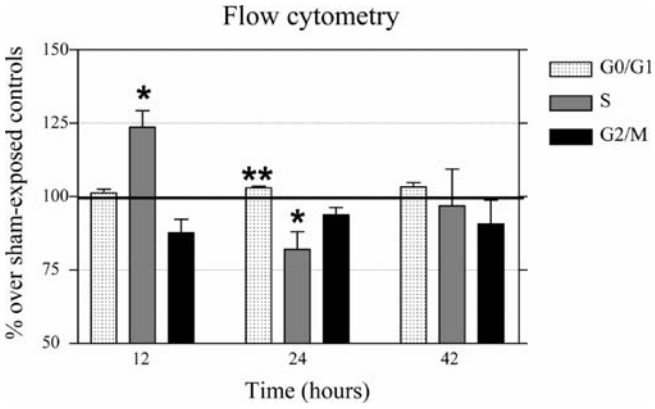

Figure 2. Percent of cells in different phases of the cell cycle after 12 or 24 h of treatment and after 18 h of post-treatment incubation in the absence of the electric stimulus (42 h). Flow cytometry analysis. Means ± SEM of three replicates. Data normalised over the respective sham-exposed controls. Student's t-test: $^{*}0.01\leq p\leq 0.05$; $^{**}0.001\leq p\leq 0.01$. Modified from ref. 8.

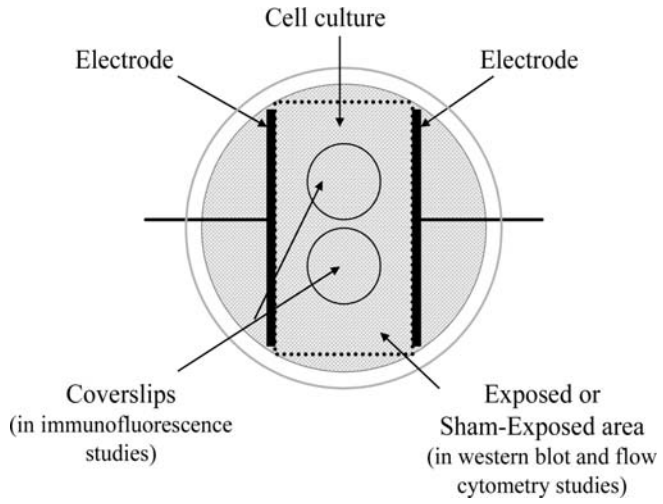

Figure 3. Pair of stainless steel electrodes inserted in a Petri dish, set for CRet stimulation or sham stimulation.

current density at the cell location within the electrode gap (Fig. 3) was calculated to be 50 μA/mm^2. Simultaneously, sham-exposed control samples were incubated under identical conditions. Subsequently, randomly selected exposed samples, together with their respective controls, were incubated for a period of 18 h post-treatment in the absence of electric stimulus (Fig. 1). Only cells grown on the dish surface homogeneously exposed to the 50 μA/mm^2 current, located within the electrode gap, were harvested for analysis (Fig. 3).

Prior to the study, a battery of tests was conducted in which a number of potential confounding factors were analyzed for artefact control (8). The following factors were investigated: influence of the electrodes, electrochemical stability of the electrodes, electrophoretic effects and thermal effects. The result confirmed that neither the electrical current nor the presence of electrodes altered the physical/chemical properties of the medium. Neither were detected significant changes in cell viability or proliferation that could be attributed to the presence of non-energized electrodes in the dishes. The 50 μA/mm^2 current did not increase the temperature of the cultures.

Immunofluorescence. For indirect immunofluorescence microscopy, cells were grown on 12 mm diameter coverslips (Fig. 3) and examined 12, 24 or 42 h after the beginning of the 24-h exposure. The cells were fixed with 4% paraformadehyde for 20 min at 4˚C and permeabilized with 95% ethanol/5% acetic acid. Cultures were incubated overnight at 4˚C with primary monoclonal antibodies against cyclin D1 (Novocastra, Newcastle, UK, at 1:50 dilution), cyclin A (Zymed, San Francisco, CA, at 1:100 dilution) cyclin B1 (Biosource, Camarillo, CA, at 1:100) or p27^{Kip1} (Biosource, at 1:100). After washing, cells were incubated with secondary antibodies conjugated to Alexa Flour 568 (Red) dye (Molecular Probes, OR) for 1 h at room temperature. Finally, coverslips were mounted on microscope slides using Non-Fade mounting medium, containing BisBenzimide H 33342 (Sigma, St. Louis, MO) for nuclei counterstaining. Cells were studied through a fluorescence microscope (Nikon Eclipse TE300) and analysed with software AnalySIS 3.1 (Soft Imaging Systems GmbH, Münster, Germany). Cells marked as positive for each of the studied proteins were counted and referred to the total cell number. In every experimental repeat, 40 fields were counted (~100 cells per field) per experimental condition. Each analysis was performed in duplicate and repeated at least 3 times.

Western blot analysis. The cells were lysed for protein extraction. The samples (100 μg protein aliquots) were separated in 10% sodium dodecyl sulfate-polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane (Amersham, Buckinghamshire, UK). The membranes were blocked with phosphate-buffered saline plus 0.1% Tween-20 and 5% dried skim milk, washed and then incubated at 4˚C overnight with mouse monoclonal antibodies against cyclin D1 (at 1:200 dilution), cyclin A (1:500), cyclin B1 (1:500) or p27^{Kip1} (1:500) respectively. ß-tubulin (1:400) was used as loading control. All proteins were purchased from Sigma. All dilutions were in PBS-Tween plus 5% dried skim milk. After washing, the membranes were incubated with horseradish peroxidase-labelled anti-mouse secondary antibody (Amersham) for 1 h at room temperature. The bands were visualized by enhanced chemiluminescence (ECL) following the manufacturer's instructions (Amersham). The blots were analyzed by densitometric assay using PDI Quantity One - 4.5.2 software (Bio-Rad, Hercules, CA). Between 3 and 10 experimental replicates were conducted for each of the proteins analyzed.

Cell proliferation. To confirm the cytostatic response observed in our previous studies, 9 experimental runs for analysis of protein expression by immunofluorescence and Western blot were selected at random. The number of live cells after 24 h of exposure or sham-exposure was quantified through hemocytometry and Trypan blue staining (0.4% in PBS).

Blind procedure and statistical analyses. All experimental procedures were carried out in a blinded manner. Data were analyzed using unpaired, two-tailed Student's t-test. Differences $p<0.05$ were considered statistically significant.

Results

Cytostatic response to treatment. At the end of the 24-h treatment, the overall cell death rate in exposed samples was not statistically different from that in the sham-exposed group, with values under 2.3% in both cases. However, the treated group showed a statistically significant decline in the number of live cells (10.5±2.2% below controls; $p<0.0001$, Student's t-test); which confirms the previously reported cytostatic response of HepG2 to the same electric stimulus.

Response of cell cycle control proteins at early stages of treatment (12 h). As shown by immunoblot and densitometric analysis cyclin A expression increased significantly by 23.5% over controls during the first 12 h of treatment (Fig. 4). A similar increase, though not significant statistically was observed in cyclin D1 expression. Fluorescence microscopy image analysis revealed the presence of cyclins D1 and A in

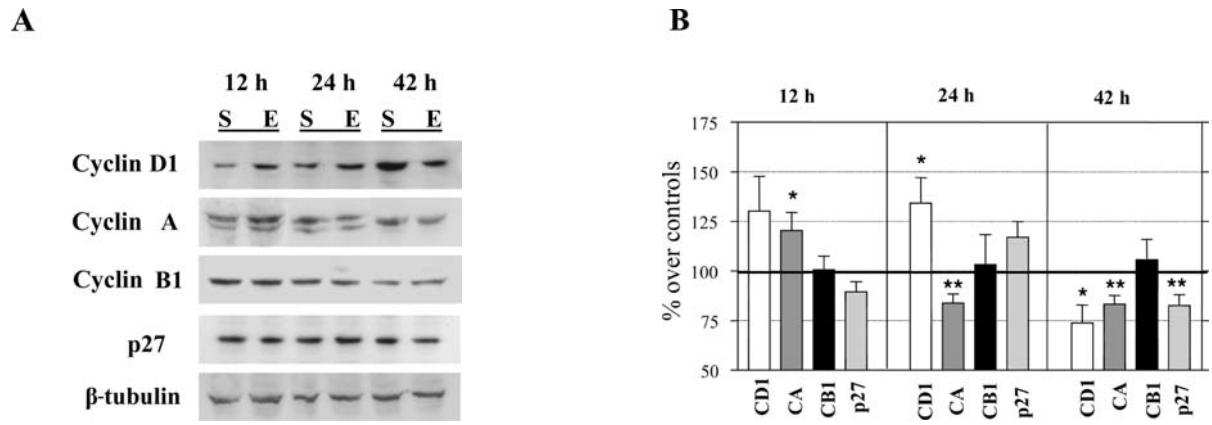

Figure 4. (A) Representative Western blots of cyclins D1, A and B1 and protein $p27^{Kip1}$ expression after 12 or 24 h of sham-exposure (S) or exposure (E) and after 18 h of post-incubation in the absence of the electric stimulus (42 h); 100 μg protein per lane. ß-tubulin was used as load control. (B) Densitometric analysis of Western blots. Means ± SEM of a minimum of three experimental replicates per protein and time interval. Data normalised over the respective controls. Student's t-test; $^{*}0.01 \leq p \leq 0.05$; $^{**}0.001 \leq p \leq 0.01$.

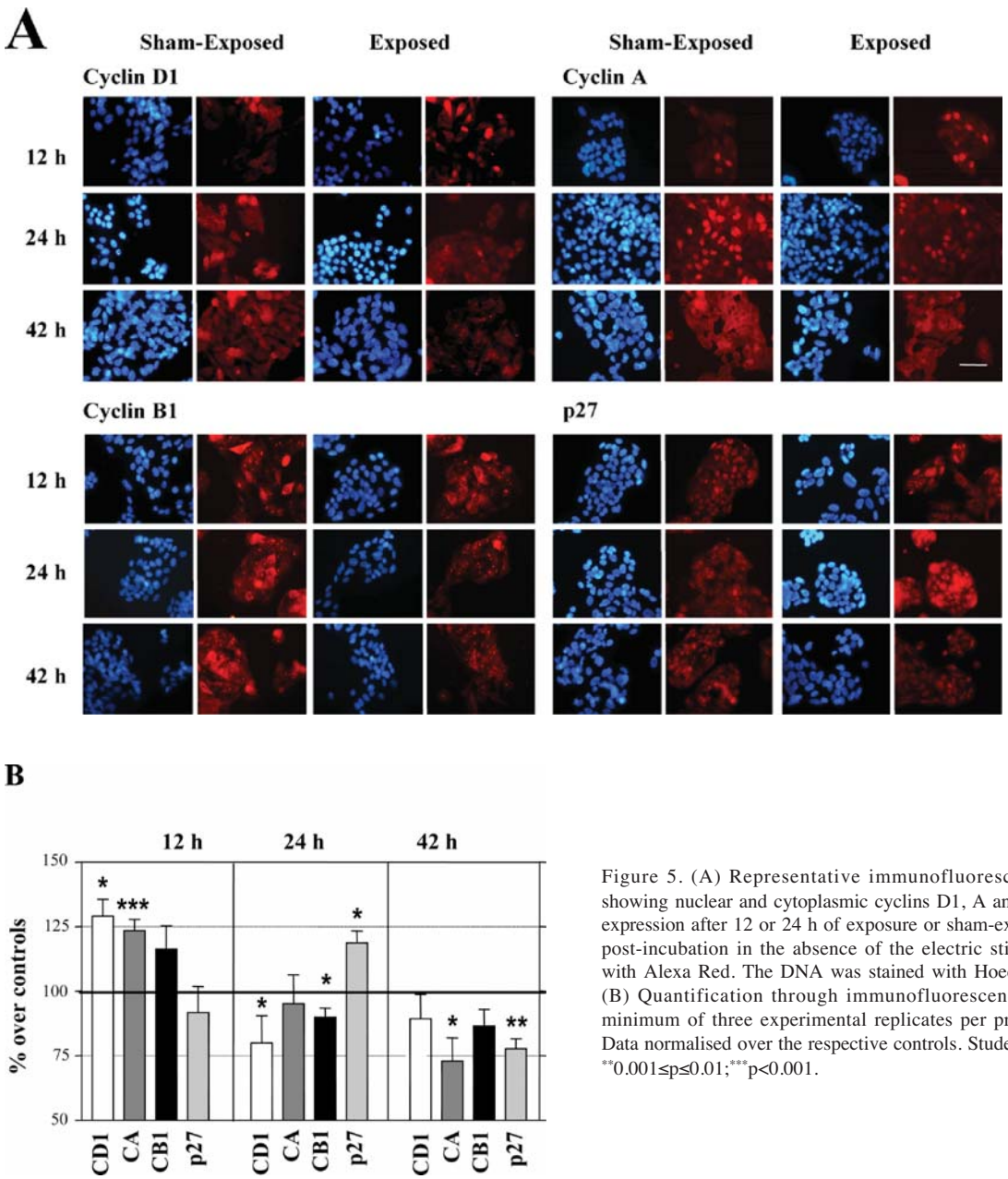

Figure 5. (A) Representative immunofluorescent photomicrographs showing nuclear and cytoplasmic cyclins D1, A and B1 and protein $p27^{Kip1}$ expression after 12 or 24 h of exposure or sham-exposure and after 18 h of post-incubation in the absence of the electric stimulus (42 h). Revealed with Alexa Red. The DNA was stained with Hoechst 33342. Bar, 50 μm. (B) Quantification through immunofluorescence; means ± SEM of a minimum of three experimental replicates per protein and time interval. Data normalised over the respective controls. Student's t-test; $^{*}0.01 \leq p \leq 0.05$; $^{**}0.001 \leq p \leq 0.01$; $^{***}p<0.001$.

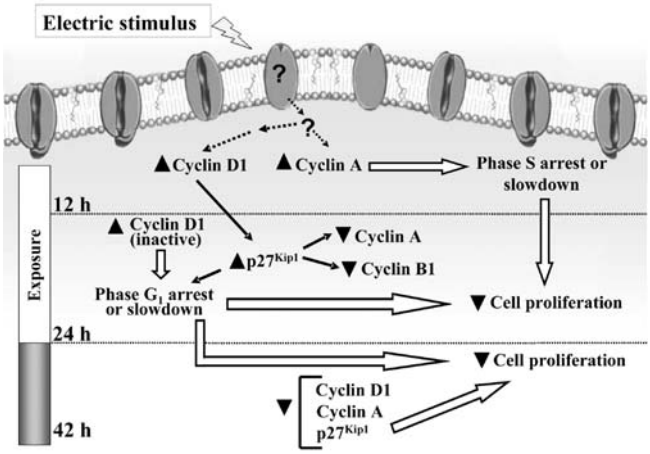

Figure 6. Proposed cascade of response to the electric treatment. Shortly after the initial, 5-min pulses cyclins D1 and A are significantly activated. The cyclin activation would result in transient arrest or slowdown of the S phase of the cell cycle at 12 h of treatment. This change in the cell cycle would act as a cell stressor that activates $p27^{Kip1}$, as observed at the end of the 24-h treatment. As a consequence, the levels of cyclins A and B1 decrease, which results in transient arrest or slowdown of the G1 phase. During the post-exposure interval, reduction of the levels of expression and activation of the cyclins A and D1 and of protein $p27^{Kip1}$ occurs. The mentioned arrest or slowdown of S and G1 phases could decelerate the proliferative rate, resulting in the observed decrease in cell number in the treated samples. Primary molecular target and pathway (dashed arrows) are unknown. ▲, overexpression and/or activation; ▼, subexpression and/or inactivation.

nuclear locations at the end of the first 12 h of stimulation, which suggests that these proteins were functionally active in the exposed samples (Fig. 5). By contrast, the expression and activation of cyclin B1 and $p27^{Kip1}$ were not affected significantly at early stages of treatment (Figs. 4 and 5).

Effects on cycle regulatory proteins at the end of the 24-h treatment. Blot densitometry assay revealed that after 24 h of treatment the total levels of cyclin A decreased significantly by 16% below controls (Fig. 4). On the other hand, immunofluorescence analysis showed reduced amount of nuclear cyclin D1 compared to controls (Fig. 5), simultaneous to increased levels of total protein (nuclear plus cytoplasmic) as revealed by blot densitometry (Fig. 4). This result suggests that even if at the end of the treatment the cyclin D1 levels remained above those in controls, the protein would have already started to inactivate at that time. As for cyclin B1, immunofluorescence revealed a statistically significant, 10% decrease in the nuclear expression of the protein at the end of the treatment, whereas blot densitometry detected no change in the protein expression compared to controls (Figs. 4 and 5). Finally, the levels of total and nuclear $p27^{Kip1}$ protein increased by 19% over controls, although only the results of the immunofluorescence analysis showed statistically significant differences with respect to control samples (Fig. 5).

Evolution of cell cycle control protein expression after treatment. After 18 additional hours of post-treatment incubation in the absence of electric stimulus (42 h) the expression of proteins cyclin D1, cyclin A and $p27^{Kip1}$ was significantly decreased with respect to controls (Figs. 4 and 5). Again, cyclin B1 assay showed no statistical differences between exposed and control samples.

Discussion

In previous studies we have reported decreased cell proliferation and partial cytostasis in HepG2 cultures treated 24 h with 0.57 MHz, sine wave electric current at 50 μA/mm^2. We proposed that such response would be mediated by arrest or slowdown of the cell cycle, affecting primarily (first 12 h of treatment) the S phase and subsequently the G1 phase, in a significant fraction of the cellular population (8). The results of the present study strongly support such hypothesis and indicate that those effects could be exerted through alterations in the expression and activation of proteins involved in cell cycle control.

Our data show that during the first 12 h of treatment cyclins A and D1 are overexpressed and activated. Deregulation of the expression of cyclins A and D could be associated with transformation processes in some cell types. For instance, overexpression of cyclin D1 has been detected in 11-13% of patients diagnosed with hepatocellular carcinoma (27) and overexpression and deregulation of cyclin A has been associated with several cancer types, including hepatocellular carcinoma (28). However, the HepG2 line, specifically, does not spontaneously overexpress cyclin A when compared to the levels in normal liver cells (29). Besides, cyclin overexpression is not always associated with malignant cellular processes and, in fact, overexpression of cyclin D1 may inhibit proliferation in a number of cell lines by delaying or blocking the G1/S phase onset (25,30). On this basis, the early overexpression of cyclins D1 and A detected in the present study could result in a lengthening of S phase during the first 12 h of treatment, followed by an arrest or slowdown of G1 phase at 24 h, as shown by the cell cycle analysis (Fig. 2), which could result in the decrease in cell proliferation observed at that time (Fig. 6).

Additionally, it has been reported that cyclin D1 acts as a target for growth factors and other exogenous factors, integrating extracellular signals into the regulation of cell cycle progression (18-20,22,31). In our study, overexpression and activation of cyclin D1 during the first 12 h of treatment might be related to a potential role of this cyclin as an intracellular target for the electric stimulus. Thus, the initial activation of cyclin D1 would act as a transducer of the exogenous electric signal to the cellular machinery, triggering processes leading to alterations in the progression of the cell cycle (Fig. 6). Although the initial signaling cascade through which cyclin D1 is activated remains unidentified, the MEK/ERK1/2 pathway could be a possible candidate. Cyclin D1 can be transcriptionally activated through Ras/ERK pathway (22), and it has been reported that in HepG2 the ectopic expression of activated MEK1, a member of the Ras/ERK pathway, induces overexpression of cyclin D1 and Cdk2, which results in increased proportion of cells in S phase without changing the fraction of cells in G1 (21). These data are consistent with our results, since after 12 h of stimulation overexpression of cyclin D1 was detected, accompanied with increased proportion of cells in S phase,

but not in G1 phase. Thus, in HepG2, the electric current could activate one or more of the MEK/ERK1/2 pathway proteins favouring or enhancing cyclin D1 transcription, thereby increasing the levels of this cyclin, as observed in our experiments.

The activation of the MERK/ERK pathway can be influenced by a number of intracellular processes and extracellular stimuli that can change the level and/or duration of ERK activation in different cell types. Although ERK activation plays a crucial role in promoting cell proliferation and survival, it has been also described that a sustained, non-transient activation of ERK can induce differentiation, senescence, cell cycle arrest and/or apoptosis in a number of cellular species (32,33). Since the electric treatment partially inhibits cell proliferation in our cultures, an overexpression or overactivation of the MEK/ERK pathway that remained during the 24 h of treatment could lead to cell cycle arrest or to differentiation of the culture. The hypothesis that the electric treatment could enhance cyclin D1 expression through MEK/ERK1/2 pathway would receive partial support from studies showing that stimulation with electric fields can activate ERK1/2 protein in various cell types (34,35).

Moreover, the lack of effect on the expression or activation of the inhibitor $p27^{Kip1}$ during the first 12 h of stimulation would indicate that $p27^{Kip1}$ does not play a significant role in the observed blocking of S phase. This would reinforce previous observations by Borel *et al* (36) and Hurley *et al* (37) that inhibitors p21 and $p27^{Kip1}$ would not intervene in the arrest and prolongation of S phase.

At the end of the 24-h treatment, the nuclear $p27^{Kip1}$ was overexpressed and activated with respect to controls, indicating that at that time $p27^{Kip1}$ would act as an inhibitor of the cell cycle (38). It has been reported that overexpression of cyclin D1 can induce increased expression of $p27^{Kip1}$ (39,40). In our experiments, the overexpression and activation of cyclin D1 detected during the first 12 h of treatment might be responsible for the activation of $p27^{Kip1}$ observed at the end of the 24-h exposure (Fig. 6). Furthermore, besides of being related to cyclin D1, $p27^{Kip1}$ has been described to intervene directly on cyclin A regulation by blocking the activation of the corresponding gene (41). Following this, the sub-expression of cyclin A detected in the HepG2 cultures at the end of electric treatment (24 h) could be due to an overexpression and activation of $p27^{Kip1}$ during the last 12 h of treatment. Thus, the treatment would induce cell cycle arrest in G1 as a consequence of the overexpression and activation of $p27^{Kip1}$ and of the cyclin A sub-expression and cyclin D1 inactivation, detected at the end of the treatment (Fig. 6). A similar effect has been described by Wang *et al* (26) who found that overexpression of $p27^{Kip1}$ causes proliferation inhibition in endothelial cells after exposure to electric fields at physiological doses.

Concerning the evolution of the cellular response during the post-exposure period, cell cycle control proteins cyclin D1, cyclin A and $p27^{Kip1}$, which were overexpressed and activated during the treatment, became subexpressed and/or inactivated 18 h after the end of electric exposure (42 h). This effect could be mediated by cellular mechanisms of adaptation or hormesis, by which cells would tend to homeostasis through processes compensatory of the undergone cytostasis (42).

In conclusion, during the first 12 h of electric stimulation, the treatment induced overexpression and/or activation of cyclins D1 and A that would result in slowing or blockage of the S phase of cell cycle in a fraction of the cellular population. This effect was not mediated by $p27^{Kip1}$. After 12 additional hours of treatment, cyclins D1, A and B1 were underexpressed below the control levels, while $p27^{Kip1}$ was overexpressed and activated. As a result, part of the cellular population could be arrested in G1 phase. This effect on the cell cycle did not stay after treatment. Thus, in the post-treatment interval, both the levels of cyclins D1, A and B1 and of the inhibitor $p27^{Kip1}$ fell below those of control samples, leading to the subsequent release of the cell cycle. As a whole, the present data indicate that electrically-induced changes in cell cycle control proteins would be responsible for the blocking or slowdown of the cell cycle phases. This would result in extended generation time of HepG2 cultures and would be a cause for the decrease in cell proliferation detected during and after treatment (between 12 h and 42 h from the start of treatment) in the present study, as well as in previous work (16). The onset of this sequential response to CRet could involve regulatory pathways like MEK/ERK1/2, one of the main signaling routes of cyclin D1 activation, which has been reported to be sensitive to the action of electric fields.

Our data show that the electric stimulus acts on proteins intervening in cell cycle regulation, which are among the targets of recently developed chemical therapies (43). Drugs such as flavopiridol or the proteasome inhibitor PS-341 induce decreased levels of cyclins D1, A and B, and arrest cells in the G1 to S phase transition, in S phase or in the G2 to M transition (44). Recent studies have reported that drugs like dulxanthone A induce apoptosis by blocking the S phase of cell cycle in the line HepG2 (45). Thus, the herein reported results indicate that *in vitro* treatment with CRet currents could induce effects that mimic the cellular response to chemicals with potential applications in oncology. The ability of this electric stimulus to alter expression and activation of cell cycle control proteins and to arrest the cell cycle, at least in a fraction of the cellular population, might open the possibility of extending the applications of CRet treatment to the field of oncology. However, the potential clinical relevance of the present results can only be determined from further, translational studies.

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