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Hexavalent chromium, a lung carcinogen, confers resistance to thermal stress and interferes with heat shock protein expression in human bronchial epithelial cells

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Abstract Exposure to hexavalent chromium [Cr(VI)], a lung carcinogen, triggers several types of cellular stresses, namely oxidative, genotoxic and proteotoxic stresses. Given the evolutionary character of carcinogenesis, it is tempting to speculate that cells that survive the stresses produced by this carcinogen become more resistant to subsequent stresses, namely those encountered during neoplastic transformation. To test this hypothesis, we determined whether pre-incubation with Cr(VI) increased the resistance of human bronchial epithelial cells (BEAS-2B cells) to the antiproliferative action of acute thermal shock, used here as a model for stress. In line with the proposed hypothesis, it was observed that, at mildly cytotoxic concentrations, Cr(VI) attenuated the antiproliferative effects of both cold and heat shock. Mechanistically, Cr(VI) interfered with the expression of two components of the stress response pathway: heat shock proteins Hsp72 and Hsp90α. Specifically, Cr(VI) significantly depleted the mRNA levels of the former and the protein levels of the latter. Significantly, these two proteins are members of heat shock protein (Hsp) families (Hsp70 and Hsp90, respectively) that have been implicated in carcinogenesis.

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Thus, our results confirm and extend previous studies showing the capacity of Cr(VI) to interfere with the expression of stress response components.

Keywords Hexavalent chromium · Lung carcinogen · Thermal shock · Stress response · Heat shock proteins

Abbreviations

BSA	Bovine serum albumin
Cr(III)	Trivalent chromium
Cr(IV)	Tetravalent chromium
Cr(V)	Pentavalent chromium
Cr(VI)	Hexavalent chromium
HSF1	Heat shock factor 1
Hsp(s)	Heat shock protein(s)
miRNAs	microRNAs
PBS	Phosphate-buffered saline
RBPs	RNA binding proteins
ROS	Reactive oxygen species
SEM	Standard error of the mean

Introduction

Hexavalent chromium [Cr(VI)] produces various adverse health effects to the skin and respiratory tract (Mancuso 1951; Mancuso and Hueper 1951; Zachariae et al. 1996; Barceloux 1999). Notably, occupational exposure to Cr(VI) compounds, as encountered in certain industries, has been firmly associated with increased rates of lung cancer (IARC 1990; NTP 2014). There is also concern for the general population, as these compounds are now widespread environmental pollutants (Armienta-Hernandez and Rodriguez-Castillo 1995; Freeman et al. 1997; Urbano et al. 2008).

Cr(VI) itself is mostly unreactive towards nucleic acids and most proteins (Fornace et al. 1981; Connett and Wetterhahn 1983; Tsapakos and Wetterhahn 1983; Salnikow and Zhitkovich 2008). Instead, several of the species arising from its intracellular reduction, namely pentavalent chromium [Cr(V)], tetravalent chromium [Cr(IV)], trivalent chromium [Cr(III)], carbon and thiyl radicals, and, possibly, reactive oxygen species (ROS), are the active carcinogens, reacting extensively and in a multiplicity of ways with these macromolecules (Cohen et al. 1993; Stearns and Wetterhahn 1994; O'Brien et al. 2003; Wang et al. 2006). Their combined chemical versatilities add a layer of complexity to the molecular mechanisms underlying Cr(VI) carcinogenicity. Accordingly, the exact nature of these mechanisms remains mostly unknown.

It is well established, though, that Cr(VI) is an inducer of oxidative, genotoxic and proteotoxic stress, through the generation of the above-mentioned reactive species, some of them potent oxidizers (Fornace et al. 1981; Tsapakos and Wetterhahn 1983; Myers 2012; Urbano et al. 2012; Li et al. 2015). Cr(VI)-induced deregulated cellular energetics, as shown by us (Rodrigues et al. 2009; Goncalves et al. 2011; Ferreira et al. 2012; Cerveira et al. 2014) and others (Liu et al. 2010; Myers et al. 2010; Molina-Jijon et al. 2011; Xiao et al. 2012a; Abreu et al. 2014), likely constitutes an additional major stressor.

Cancer cells are remarkably resistant to the high levels of stress inherent to malignant progression (Hanahan and Weinberg 2011). Among established intra and extracellular stressors are tumor hypoxia, acidosis, nutrient deprivation, redox deregulation, imbalance in protein production, the presence of mutant oncoproteins and deregulated cellular energetics (Wondrak 2015). Interestingly, it is now becoming increasingly clear that cancer cell survival relies on an array of genes and functions that are not inherently tumorigenic, but rather components of the cellular machinery normally employed to respond to stress, most prominently the stress response and the antioxidant defense (Sun 1990; Morimoto 1993; Jolly and Morimoto 2000; Dai et al. 2007). Importantly, there is now some indication that the molecular pathways that underpin these protective mechanisms shared by all cells may constitute a selective liability for malignant cells—as opposed to normal cells, as the latter are not constantly exposed to abnormally high levels of extracellular and intracellular stressors (Raj et al. 2011). Thus, this 'non-oncogene addiction' model of tumorigenesis opens new avenues for the development of novel therapeutic strategies against cancer.

The cytoprotective effects of the stress response are essentially due to the action of the heat shock proteins (Hsps), which act as molecular chaperones and anti-apoptotic proteins, among other functions (Csermely and Yahara 2002; Takayama et al. 2003). Hsps are overexpressed in response to stress, a process that, in mammals, is orchestrated mostly by the transcription

factor heat shock factor 1 (HSF1) (Dai et al. 2007). Permanent cellular stress might explain the increased HSF1 activity and elevated levels of most Hsps observed in several types of tumors (Ciocca et al. 2013). Various Hsps have now been implicated in different hallmarks of cancer, including epithelial cell migration, tumor invasiveness and resistance to chemotherapy (Ciocca et al. 2013).

It is conceivable that the stress response plays a major protective role in those cells that resist Cr(VI) exposure, but this hypothesis remains largely unexplored. There are a few reports in the literature on the impact of Cr(VI) on the stress response, but these are inconclusive: while there are some data suggestive of stress response induction by Cr(VI) (Delmas et al. 1998; Ye and Shi 2001; Izzotti et al. 2002; Lei et al. 2008; Zhang et al. 2010; Lee and Lim 2012), there are also data pointing to the opposite (Andrew et al. 2003; Banu et al. 2011; Xiao et al. 2012b). In this study, we show that Cr(VI) protects human bronchial epithelial cells against acute thermal shock and alters the expression of heat shock proteins Hsp72 and Hsp90α, at the mRNA and protein levels, respectively.

Materials and methods

Chemicals and biochemicals

LHC-9 medium (Gibco®) was obtained from Life Technologies™ (Carlsbad, CA, USA). ELISA kits were obtained from Enzo Life Sciences (New York, USA). Kits and reagents used in mRNA quantification were purchased either from Bio-Rad (Amadora, Portugal) or Qiagen (Hilden, Germany). Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (Sintra, Portugal).

Cell culture

The BEAS-2B cell line (ECCAC no. 95102433) was purchased from the European Collection of Cell Cultures (Salisbury, UK). Cultures were established in tissue culture vessels containing LHC-9 medium (ca 0.2 mL/cm^2 of growth surface) and were maintained at 37 °C, in a humidified atmosphere of 95% air/5% CO_2. All tissue culture vessels were pre-coated with a mixture of gelatin (type B) and bovine serum albumin (BSA).

Cr(VI) treatments

Cr(VI) treatments were carried out 24 h after seeding, using a 50 μM potassium dichromate ($K_2Cr_2O_7$) solution, prepared in ultrapure water and filter-sterilized before its first use. In all experiments, all cultures, including controls [0 μM Cr(VI)], were established and processed in parallel and all received the same volume of the addition vehicle (ultrapure water).

Determination of doubling times

Doubling times were calculated from plots of the natural logarithm of the number of live cells in culture against time in culture. For each condition tested, triplicate cultures per time point were established in 6-well plates, at a seeding density of 2000 cells/cm^2. At the desired time points, the corresponding cultures were washed with phosphate-buffered saline (PBS) and were subsequently covered with 300 μL of a 0.25% (w/v) trypsin solution. Following a brief incubation at room temperature, a similar volume of PBS was added. After resuspension, cells were immediately sat on ice. For cell counting, cell suspensions were thoroughly mixed and a small volume of the resulting suspension was mixed with a 0.4% (w/v) Trypan blue solution. Cell counts were performed under an inverted microscope, using a hemocytometer. Cells dyed blue were scored as dead. All cultures assayed were in the exponential phase of growth, as confirmed by the linearity of the above-mentioned plots.

Induction of thermal shocks and assessment of their effects on cell proliferation

For each condition tested, cultures were established, either in triplicate or tetraplicate, in 6-wells plates, at a seeding density of 4000 cells/cm^2. Twenty-four hours post Cr(VI) addition, cultures were removed from the incubator and subjected to thermal shock. For cold shock induction, spent medium was replaced by cold medium (4 °C) and cultures were immediately returned to the incubator, where they remained for an additional 24 h. Heat shock induction was achieved by replacing spent medium with fresh medium pre-warmed to 43 °C and maintaining these cultures at 43 °C (in a water bath) for 1 h. Afterwards, cultures

were returned to the incubator where they remained for 24 h.

Proliferation rate, here defined as the ratio of the number of live cells at 24 h post-shock to the number of live cells at the time of shock, was the metric chosen to gauge the effects of thermal shock. The magnitude of these effects was evaluated by comparing the ratios obtained for stressed cultures with those obtained for their non-stressed counterparts (i.e., cultures that were established and processed in parallel, received the same amount of Cr(VI) and/or ultrapure water, but were not subjected to thermal shock).

Quantification of individual protein levels by ELISA

Intracellular levels of individual proteins were determined, using ELISA kits, in cell extracts prepared 48 h after Cr(VI) addition. Cell extraction and protein quantification were performed according to the manufacturer's instructions. For the quantification of Hsp72 and HSF1, cells were seeded at 6000 cells/cm^2 in 175 cm^2 flasks, whereas they were seeded at 4000 cells/cm^2 in 25 cm^2 flasks for Hsp90α quantification. Results were normalized against total protein levels, which were determined in the corresponding cell extracts using the Bio-Rad protein assay dye reagent concentrate, with BSA as the standard.

Quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

To determine mRNA levels, duplicate cultures for each condition tested were established in 75 cm^2 flasks, at a seeding density of 8000 cells/cm^2. Forty-eight hours after Cr(VI) addition, total RNA was extracted, quantified and reverse transcribed using, respectively, the Aurum™ Total RNA Mini Kit (Bio-Rad), a Nanodrop 2000 spectrophotometer (ThermoScientific, Waltham, MA, USA) and the iScript™ cDNA Synthesis Kit (Bio-Rad). qPCR was performed using the SsoFast™ EvaGreen® Supermix, in a CFX96 real time-PCR system (Bio-Rad). Additional details about the reaction settings, quality control and normalization, as well as a description of the primers, can be found in Electronic Supplementary Material 1.

Statistical analysis

For each parameter analysed, at least three independent experiments were performed. All results are presented as mean ± SEM (standard error of the mean). The statistical significance of the differences from the control was assessed by Student's paired *t* test or by one-way analysis of variance (ANOVA), followed by *Dunnett's* multiple comparison test, using GraphPad Prism 5.00 software. Differences with $p < 0.05$ were considered statistically significant.

Results and discussion

The aim of this study was twofold: to test the hypothesis that incubation with Cr(VI) protects cells from subsequent stresses and to gain further insight into the impact of Cr(VI) on the stress response.

In the context of carcinogenesis, inhalation is the major route of Cr(VI) entry into the body. Inhaled Cr(VI) insoluble particles deposit preferentially in the bifurcations of the bronchi, where they accumulate (Ishikawa et al. 1994b). Exposure of bronchial epithelial cells to the soluble Cr(VI) that is slowly released from these particles produces atypical lesions, from which malignant neoplasms may develop (Ishikawa et al. 1994a). Thus, to address the issue of physiological relevance, human bronchial epithelial cells, the main targets of Cr(VI) carcinogenicity, were used throughout this study. Exposure regimens were of low cytotoxicity, in line with the combined findings of several groups concerning occupational exposures to Cr(VI) (Tsuneta et al. 1980; Raithel et al. 1993; Sunderman 2001; Caglieri et al. 2008).

Acute thermal shock was used as a model of cellular stress and its impact was evaluated in terms of cell growth inhibition, regarded as one of its strongest effects (Kuhl and Rensing 2000; Al-Fageeh and Smales 2006). To this end, the proliferation rates of stressed cultures over a 24 h period post thermal shock were compared with those of their non-stressed counterparts, established and processed in parallel. The potential protective action of Cr(VI) against this type of stress was assessed by pre-incubating cells with this carcinogen 24 h prior to thermal shock induction.

The impact of Cr(VI) on the stress response was monitored in terms of the expression, at the transcript and protein levels, of Hsp72, Hsp90α and HSF1. Our choice of Hsp72 was based on the observation that this stress-inducible isoform, as well as various other members of the Hsp70 family, is frequently found overexpressed in several cancers, correlating with poor prognosis (Ciocca and Calderwood 2005; Juhasz et al. 2013), which might be related to increased invasive and metastatic capacities and with resistance to chemotherapy (Budina-Kolomets et al. 2015). As to Hsp90α, it is believed that augmented levels of this protein increase the stability of mutated signaling proteins, thus potentiating the metabolic shift and invasiveness observed in tumors (Whitesell and Lindquist 2005). HSF1 is essential for the proliferation of many cancer cell types (Mendillo et al. 2012).

Cr(VI) stimulates cellular proliferation

Previous studies from our group suggested increased proliferation rates for BEAS-2B cells exposed to mild Cr(VI) concentrations (0.1–2 μM) (Costa et al. 2010; Ferreira et al. 2012; Cerveira et al. 2014). By determining the doubling times of Cr(VI)-exposed cultures, we now confirmed that Cr(VI) produces a concentration-dependent increase in proliferation rate (Fig. 1). Thus, those cells that resisted Cr(VI) exposure either gained or already possessed a proliferative advantage. Either way, it is clear that Cr(VI) exerts a selective pressure on BEAS-2B cell populations. In this respect, it is worth mentioning a study conducted in the early 1980s where low levels of Cr(III)-DNA adducts in single-stranded DNA were associated with faster replication rates at the expense of decreased fidelity (Snow and Xu 1991). More recently, it was shown that, in the p53 gene, whose protein product is responsible for DNA damage-induced cell cycle arrest and apoptosis (Levine 1997), Cr(III)-DNA adducts formed preferentially in regions that are frequently mutated in lung cancer (Arakawa et al. 2006). Thus, repeated exposure to low doses of Cr(VI) might gradually increase the rate of cell division with a concomitant decrease in replication fidelity. This would lead to an accumulation of mutations, further augmenting the replicative potential of the exposed cells, thus entering in a positive feedback loop.

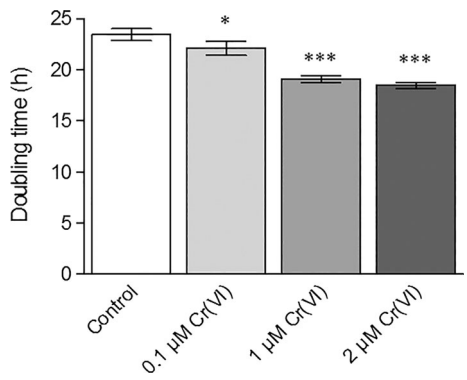

Fig. 1 Cr(VI) concentrations in the low micromolar range stimulate human bronchial epithelial cell proliferation. Doubling times were obtained from plots of the logarithm of the number of live BEAS-2B cells versus time in culture. Each experiment was performed with triplicate cultures. The linearity of the plots confirmed that all cultures were in the exponential phase of growth throughout the experiments. Results are presented as mean ± SEM of three independent experiments. Statistical analysis was performed by a one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test, using GraphPad Prism software (version 5.00). *, $p < 0.05$; ***, $p < 0.001$

Cr(VI) confers resistance against acute thermal shock

As can be appreciated in Fig. 2a, the inhibition of proliferation resulting from cold shock was significantly attenuated in cultures that were pre-incubated with 1 μM Cr(VI). The lowest Cr(VI) concentration tested (0.1 μM) also elicited a slight (but not statistically significant) attenuation. No protection was observed when cells were pre-incubated with 2 μM Cr(VI).

Since the aim of this study was to explore the hypothesis that incubation with Cr(VI) protects cells from further stress and not to carry out a comprehensive investigation on this protective effect, all subsequent experiments were conducted with a single Cr(VI) concentration, thus considerably reducing the overall cost of the study. Taking into account the results just described, as well as the cytotoxicity data obtained in previous studies by our group (Costa et al. 2010; Cerveira et al. 2014), as well as by the group of Caglieri and co-workers (Caglieri et al. 2008), 1 μM Cr(VI) was the concentration chosen. Importantly, Caglieri and co-workers showed that, in the cell line used throughout this study, the intracellular chromium levels after a 24 h exposure to this concentration were

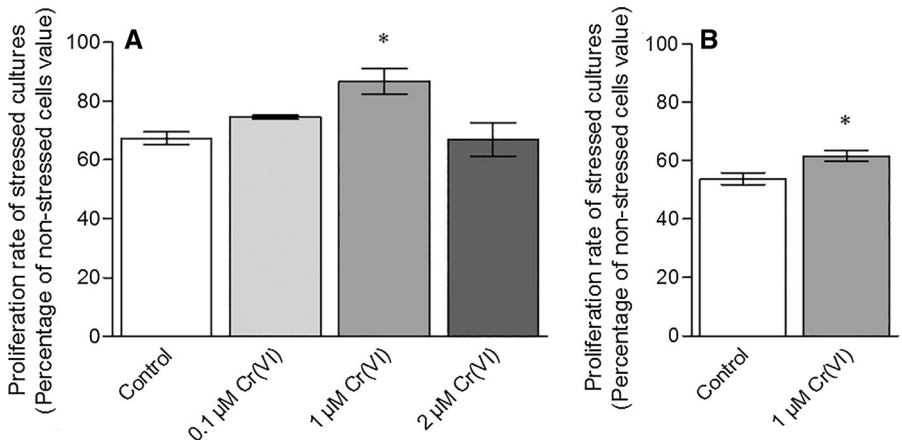

Fig. 2 Exposure of human bronchial epithelial cells to 1 µM Cr(VI) confers resistance against thermal shock-induced growth arrest. Acute **a** cold shock and **b** heat shock induction are described under Materials and Methods. Proliferation rates were defined as the ratio of the number of live BEAS-2B cells at 24 h post-shock to the number of live cells at the time of shock. Percentage values were calculated by dividing the ratio obtained for stressed cultures by that obtained for their non-stressed counterparts (i.e., cultures that were established and processed in parallel, received the same amount of Cr(VI) and/or ultrapure water, but were not subjected to thermal shock). Results are presented as mean ± SEM of three independent experiments. Data were analyzed with GraphPad Prism software (version 5.00) using **a** a one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test or **b** the Student's paired *t* test: *, $p < 0.05$

comparable to those observed in lung tissue of chromate workers (Caglieri et al. 2008).

Figure 2b shows that the protection conferred by 1 µM Cr(VI) was not restricted to cold shock. Indeed, although protection against heat shock was not as pronounced as that observed against cold shock, the effect was statistically significant and consistently observed in all three independent experiments performed. One future goal is to determine whether Cr(VI) also increases resistance to stresses more relevant in the context of carcinogenesis, such as hypoxia and nutrient deprivation.

Cr(VI) interferes with the expression of Hsp72 and Hsp90α

Next, the effect of Cr(VI) on the expression levels of key elements of the stress response was determined. At the protein level, Cr(VI) had essentially no effect on the expression of Hsp72, but it did cause a significant decrease (ca. 60%) in the expression of Hsp90α (Fig. 3a). As expected, HSF1 levels were essentially unchanged. In fact, as is the case with other transcription factors, HSF1 activity is likely mostly regulated at the post-translational level. It is known, namely, that HSF1 activity is dependent on phosphorylation status (Pirkkala et al. 2001) and on the redox status of two cysteine residues within its DNA-binding domain (Ahn and Thiele 2003). Considering previous reports showing the ability of Cr(VI) to oxidize the thiol groups of thioredoxins and peroxiredoxins (Myers et al. 2008; Myers and Myers 2009), two protein families that play a critical role in thiol redox control and, ultimately, cell survival (Nonn et al. 2003; Chang et al. 2004), one may envision that Cr(VI) might modulate HSF1 activity via oxidation of the above-mentioned HSF1 cysteine residues. Thus, in the future, it will be important to evaluate the intracellular levels of phosphorylated HSF1 and the oxidation status of this protein in Cr(VI)-exposed cultures.

The observed decrease in Hsp90α protein levels upon Cr(VI) exposure was in line with earlier reports in Cr(VI)-exposed primary cultures of rat granulosa cells (Banu et al. 2011) and in a cell line derived from human embryonic hepatocytes (Xiao et al. 2012b). Nonetheless, considering our observation that Cr(VI) conferred resistance against thermal shock, this result might seem somewhat intriguing. It must be noted, though, that resistance to thermal shock was evaluated based on changes in cell proliferation over a period of 24 h post-shock (starting 24 h after Cr(VI) addition), whereas Hsp protein levels were determined at a single time point–48 h after Cr(VI) addition. Previous studies on the kinetics of Hsp expression have shown that

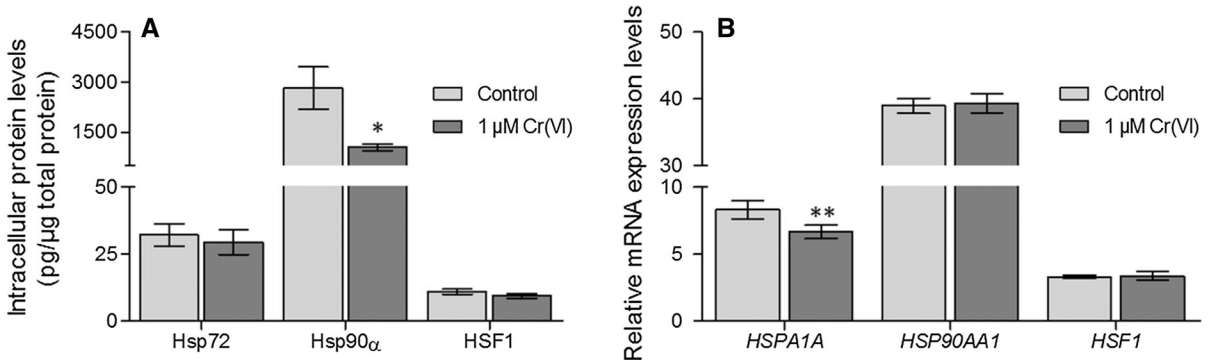

Fig. 3 Cr(VI) interferes with the expression of heat shock protein genes in human bronchial epithelial cells. **a** Individual protein levels were determined, using ELISA, in extracts of BEAS-2B cells prepared 48 h after Cr(VI) addition. The results were normalized to total protein levels. Displayed values are the mean ± SEM of five (Hsp72), six (Hsp90α), or two (HSF1) independent experiments. **b** Individual mRNA levels were determined, by RT-qPCR, in extracts of BEAS-2B cells prepared 48 h after Cr(VI) addition. The results were normalized to the mRNA levels of *YWHAZ* and *PI4 KB* and are presented as mean ± SEM of four independent experiments. Experimental data were analyzed with GraphPad Prism software (version 5.00), using Student´s paired *t* test. *, $p < 0.05$; **, $p < 0.01$. More details can be found in Electronic Supplementary Material 1. Gene symbols: *HSP90AA1*, heat shock 90 kDa protein 1 alpha; *HSPA1A*, heat shock 72 kDa protein; *HSF1*, heat shock factor 1

overexpression of these proteins following sublethal thermal stress is a transient event (Wang et al. 2003; Diller 2006). More recently, a study evaluating transcript levels in grape leaves subjected to heat shock revealed that, for some Hsp isoforms, the upregulation observed immediately after shock was followed by a decline to expression levels lower than the controls during the recovery phase (Liu et al. 2012). Thus, it is possible that Hsp90α protein levels did increase at some point post-Cr(VI) addition, decreasing at a later time down to values lower than those observed in the controls.

In the two above-mentioned studies, Cr(VI)-exposed cells also exhibited decreased Hsp70 protein levels, whereas the opposite outcome, i.e., increased Hsp70 protein levels, was observed by a different group studying Cr(VI)-exposed embryonic murine liver cells and in ICR mouse liver tissue (Lee and Lim 2012). The different outcomes are likely due to differences in the model system and exposure regimen used. Also, not all studies specified the Hsp isoform evaluated.

As can be appreciated in Fig. 3a and b, the relative amounts of HSF1, HSP72 and Hsp90α mRNA and protein found in control cells follow the same trend. However, in contrast with the measured protein levels, the mRNA levels of *HSPA1A*, which encodes Hsp72, were decreased, and those of *HSP90AA1*, which encodes Hsp90α, remained unaltered (Fig. 3b). Decoupling of mRNA and protein steady state levels has been frequently observed (Greenbaum et al. 2003; Bauernfeind and Babbitt 2017) and may result, for instance, from the actions of critical post-transcriptional regulators, such as RNA binding proteins (RBPs) and microRNAs (miRNAs) (Glisovic et al. 2008; Janga and Vallabhaneni 2011). Moreover, post-transcriptional protein modifications may affect protein stability and turnover (Sadoul et al. 2008; Doherty et al. 2009), further contributing to different profiles of protein and mRNA expression. In our study, different kinetics of the transcriptional and translational programs activated by Cr(VI) treatment, as well as different rates of mRNA and protein degradation, might have also contributed to the decoupling.

The number of studies assessing the impact of Cr(VI) on the mRNA levels of members of the Hsp70 and Hsp90 families is very small and, unfortunately, none of them assessed the impact on the corresponding protein levels. The only study found in the literature addressing Cr(VI) effects on *HSP90AA1* expression also employed BEAS-2B cells and reported downregulation (Andrew et al. 2003). There is also a report of unchanged *HSP-70* mRNA levels in HepG2 cells, but the authors did not specify the isoform evaluated (Majumder et al. 2003). *HSPA1A* mRNA levels were found increased upon Cr(VI) acute exposure in rat lung tissue (Izzotti et al. 2002) and in HT29 and HepG2 cells (Delmas et al. 1998). Once again,

comparisons are made difficult by significant differences in terms of the cell line employed, Cr(VI) concentration, duration of the exposure and/or isoform assessed.

Cr(VI) interferes with the expression of ATM and ATR, but not with the expression of BRCA1, MYC and TP53

To further test the 'non-oncogene addiction' model for Cr(VI) carcinogenesis, the effects of the same Cr(VI) exposure regimen on the mRNA levels of two important regulators of the DNA damage response, *ATM* and *ATR* (Shiloh 2001), as well as on those of three well-studied cancer genes, *BRCA1*, *MYC* and *TP53,* were investigated. The connections between these five proteins and the stress response can be briefly summarized as follows. The modulation of ATM and ATR by Hsp90 inhibitors suggests a role for Hsp90 in the stabilization of these proteins and/or of some of their upstream regulators (Cerchietti et al. 2009; Makhnevych and Houry 2012). BRCA1 is a known client protein of Hsp90 and loss of Hsp90 function abolishes BRCA1-dependent DNA DSB repair (Stecklein et al. 2012). The stabilization and transcriptional activity of p53 are also dependent on the chaperone activity of Hsp90 (Muller et al. 2004; Walerych et al. 2004). Both ATR and p53 form complexes with HSF1 which modulate the transcriptional activity of p53 in response to DNA damage (Logan et al. 2009). It was also suggested that HSF1 is required for p53 nuclear translocation (Li et al. 2008). Finally, by modulating of the activity of Hsp gene promoters, p53 and c-Myc regulate their expression: p53 represses the promoters, while c-Myc stimulates their activity (Calderwood et al. 2006).

As can be appreciated in Fig. 4, Cr(VI) statistically changed the mRNA levels of *ATM* and *ATR*, with the first being downregulated and the second upregulated. The importance of these changes that, although very small, were consistently observed in all four independent experiments performed remains, for the time being, unknown. Interestingly, the same Cr(VI) exposure regimen did not produce statistically significant changes in the mRNA levels of *BRCA1*, *MYC* and *TP53*. Thus, there is some possibility that early lung carcinogenesis induced by Cr(VI) may co-opt genes not normally viewed as drivers of carcinogenesis.

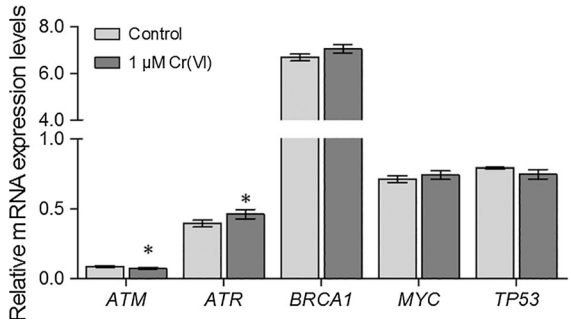

Fig. 4 Cr(VI) alters the expression of the stress-induced genes *ATM* and *ATR* in human bronchial epithelial cells. Individual mRNA levels were determined, by RT-qPCR, in extracts of BEAS-2B cells prepared 48 h after Cr(VI) addition. The results were normalized to the mRNA levels of *YWHAZ* and *PI4 KB* and are presented as mean ± SEM of four independent experiments. Data were analyzed with GraphPad Prism software (version 5.00), using Student's paired *t* test: *, $p < 0.05$. More details can be found in Electronic Supplementary Material 1. Gene symbols: *ATM*, ataxia telangiectasia mutated; *ATR*, ataxia telangiectasia and Rad3-related protein; *BRCA1*, breast cancer 1 susceptibility protein; *MYC*, v-Myc avian myelocytomatosis viral oncogene homolog; *TP53*, tumour protein p53

Conclusions

In their progression to a fully neoplastic phenotype, cells must resist a multitude of intracellular and extracellular stresses. One of the aims of this exploratory study was to investigate whether the initial stresses produced by Cr(VI) exposure conferred the surviving cells an increased resistance to subsequent stresses. Our results do show that Cr(VI) exposed cells were more resistant to thermal shock, used here as a model stressor, than their non-exposed counterparts.

This study also confirmed and extended earlier reports that Cr(VI) interferes with the expression of key elements of the stress response (although, by itself, it does not fully support an induction of the canonical stress response). Future studies involving different exposure regimens and assessing Hsp levels at different times of the exposure and/or recovery phases, as well as assessing additional components of the stress response will undoubtedly shed more light on the involvement of the stress response in Cr(VI)-acquired resistance to stress and, ultimately, on the mechanisms underlying Cr(VI)-induced carcinogenesis.

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